2009

THE INTERPLAY BETWEEN THE EXPRESSION AND FUNCTIONS OF WNT13 ISOFORMS DURING APOPTOSIS IN BOVINE AORTIC ENDOTHELIAL CELLS

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine
at the University of Kentucky

By
Tao Tang

Lexington, Kentucky

Director: Dr. Catherine D. Mao, Assistant Professor of Nutritional Sciences

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Wnt proteins are crucial for development/homeostasis by controlling cell fate including apoptosis (Moon RT et al. 1997). Three humanWnt13 isoforms were identified: the secreted Wnt13A, mitochondrial Wnt13B, and nuclear Wnt13C forms; and nuclear Wnt13 had an increased sensitivity to LPS/TNF-induced apoptosis in primary endothelial cells (EC); both Wnt13B and C mRNA contain two start codons (AUG+1 and +74), but the same protein encoded from AUG+74 by Wnt13C was expressed lower than Wnt13B (Struewing IT et al.2006). We hypothesize that during EC apoptosis, the nuclear Wnt13C expression is regulated translationally; nuclear Wnt13 favors apoptosis through regulating the activity/expression of apoptosis-related factors; Wnt13 isoforms may have differential effects on EC apoptosis and apoptosis-related factors.

1. The protein levels, but not the mRNA levels of Wnt13C were induced by apoptosis-inducers. And the Myc-tag insertion at the AUG+1 in Wnt13C mRNA inhibited its expression, indicating the RNA sequences/structures are critical. Therefore, nuclear Wnt13C is regulated during apoptosis at translational levels.

2. Nuclear Wnt13 increased caspase-3/7 expression with/without LPS, followed by an increase in LPS-induced caspase-3/7 cleavage; and nuclear Wnt13 upregulated the pro-apoptotic Bcl-2 family member Bim expression, suggesting that nuclear Wnt13 increased caspase activation through upregulating caspase and Bim expression. Wnt13 isoforms increased EC apoptosis with different strengths: nuclear > mitochondrial > secreted forms.

3. Both caspase-3 and Bim are FOXO target genes; and nuclear Wnt13 increased the nuclear localization of FOXOs, suggesting increased FOXO activity. Nuclear Wnt13 also upregulated *SOD2*, another FOXO target gene related to oxidative stress-resistance.

Nuclear Wnt13 did not increase FOXO activity at the *SOD2* promoter, but increased the SOD2-intron 2 element luciferase activity upon LPS, where a novel putative FOXO site was found, implying intron 2 may be responsible for enhanced *SOD2* transcription by nuclear Wnt13.

Altogether, our results pinpoint the interplay between the expression and functions of Wnt13 forms during EC apoptosis, forming a positive cycle further facilitating the apoptotic program completion, which is important for EC homeostasis.

KEYWORDS: Translational regulation, caspases, FOXOs, *SOD2*

transcription, EC homeostasis

Tao Tang

Nov. 4, 2009

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

1.1 Wnts and Wnt signaling

1.1.1 Overview

1.1.1.1 Brief history

In 1982, Nusse R and Varmus ER firstly identified a mouse proto-oncogene called *Int-1* as a preferential integration site in the host genome for insertional activation by mouse mammary tumor virus (MMTV) in mammary tumors (Nusse R et al. 1982). When sequenced, *Int-1* was found to encode a novel secreted glycoprotein (van Ooyen A et al. 1984). The Drosophila homolog of *Int-1* (*Dint-1*) turned out to be identical to a segment polarity gene, *wingless (Wg)*, which mutation led to the lack of wings and halteres in adult flies (Sharma RP et al. 1976, Rijsewijk F et al. 1987a). In 1991, Nusse R et al proposed a new nomenclature to call *Int-1* and related genes Wnt, which is an amalgam of Wingless and Int (Nusse R. 1991). Wnt1 was shown to induce morphological transformation in cultured cell lines, including C57MG mammary epithelial cells (Brown AM et al. 1986; Jue SF et al. 1992), RAC311 mammary cells (Rijsewijk F et al. 1987b), PC12 pheochromocytoma cells (Bradley RS et al. 1993), and C3H 10T1/2 fibroblasts (Bradbury JM et al. 1994). Then people extended to all the other Wnts, and found that different Wnts have distinct ability in transforming activity; hence the Wnt family members were divided into two groups based on this biological activity: Wnt1, 3a and 7a are highly transforming members, while Wnt 4, 5a and 11 are non-transforming members (Wong GT et al. 1994). In the late 1980s and early 1990s, many genes were discovered via screen of *Drosophila* mutations displaying segment polarity

defects (Nüsslein-Volhard C et al. 1980; Eberl DF et al. 1988), which helped the identification of components of the Wnt signaling cascade, such as the cytoplasmatic Disheveled (Klingensmith J et al. 1994) and nuclear signal transducers Armadillo/β-catenin (Wieschaus E et al. 1987), and then the epistatic relationship between these molecules was characterized showing that they comprise the Wnt canonical signaling pathway (Klaus A et al. 2008). So people later on prefer using the terms canonical Wnt (initiating β-catenin/T cell factor pathway) and non-canonical Wnt (Wnt-Plannar cell polarity pathway or Wnt- Ca^{2+} pathway) to classify the Wnt members (Herman M. 2001; van Amerongen R et al. 2006). Today the Wnt picture has been greatly enriched by the discovery of new members, receptors, coreceptors, target genes, agonists, antagonists, biological functions of Wnt signaling, as well as the involvement in human diseases, which timeline has been reviewed by Klaus A et al (Klaus A et al. 2008).

1.1.1.2 Wnt protein family

Wnt proteins are highly conserved throughout the animal kingdoms, with Wnt homologs found in genomes of human, mouse, xenopus, chicken, zebrafish, *Drosophila*, *C. elegans* and even Cnidaria (van Amerongen R et al. 2008 and Cadigan KM et al. 1997). There are 19 Wnt proteins in the mammalian genome which can be divided into 12 conserved subfamilies based on evolutionary relationship, and they are expressed in temporal-spatial patterns during development (Clevers H. 2006).

Most Wnt proteins have around 350 amino acids in sequence with a molecular weight of about 40KD (Coudreuse D et al. 2007). Wnts feature a signature Wnt motif (C-K-C-H-G-[LIVMT]-S-G-S-C), as well as 23-25 conserved cysteine residues and several N-glycosylation sites (Zhai L et al. 2004; Coudreuse D et al. 2007). Thus, one post-translational modification that Wnts undergo is N-glycosylation, and Wnt1, 3a, 3a, 5b, 6 and 7b have been shown to enter the endoplasmic reticulum (ER) and to be N-glycosylated (Smolich BD et al. 1993). Another post-translational modification is lipid modifications (addition of palmitate at cysteine residues or palmitoleic acid at serine residues) which contribute to the insolubility of Wnts (Kikuchi A et al. 2007). So far, Wnt1 (Franch-Marro X et al. 2008), 3a (Willert K et al. 2003) and 5a (Kurayoshi M et al. 2007) have been reported to undergo lipid modifications. And this hydrophobic feature of Wnts confers a strong affinity for cell membrane; for example, Drosophila Wnt-1 (Wg) was shown to be converted to a membrane-anchored (more specifically, lipid raft-targeted) protein after lipid modification (Zhai L et al. 2004). Both N-glycosylation and lipid modification have been shown critical for the secretion and action of Wnt3a (Komekado H et al. 2007; Takada R et al. 2006; Kurayoshi M et al. 2007). However, for Wnt5a, palmitoylation is critical for its activity, but N-glycosylation is necessary for only the secretion (Kurayoshi M et al. 2007). For Drosophila, palmitoylation has been found to regulate the activity of Wg (Galli LM et al. 2007), but WntD does not require lipid modification for its secretion and function (Ching W et al. 2008). When Wnt enter into ER, Wnt undergoes N-glycosylation and then acylation by membrane-bound O-acyl transferases PORC (Porcupine for Drosophila); subsequently, Wnt is transported into the Golgi apparatus, where further glycosylations occur, and then Wnt (Wg or XWnt4) is

routed to plasma membrane accompanied by Wntless (a conserved membrane protein that is dedicated in Wnt secretion) to be finally secreted from cell surface in a mature and active form (Zhai L et al. 2004; Bänziger C et al. 2006; Kikuchi A et al. 2007; Kim H et al. 2009). However, this model does not apply for all the Wnts. For example, not all of the Wnts are secreted, e.g. Wnt13B and C are intracellular Wnts (Streuwing IT, 2006).

Wnt1, Wnt3a, and Wnt8 are usually believed to be canonical Wnts while Wnt5a and Wnt11 are usually called non-canonical Wnts, based on which signaling pathways they initiate, respectively. However, it is difficult to categorize Wnts due to the lack of clear distinction among all the Wnts by using this classification, and there are increasing evidence to support that Wnt proteins *per se* are not intrinsically canonical or noncanonical but that the pathways that Wnt ligands activate is determined by the context of receptors (van Amerongen R et al. 2008), and Table 1 demonstrated the details of individual Wnt that binds which specific frizzled (Fz) receptors and then initiates specific signaling pathways.

1.1.1.3 Receptors and ligands in Wnt signaling

1.1.1.3.1 Frizzled (Fz) receptors

The interaction between Wnts and their receptors on the cell surface is the first step in transducing the extracellular signal into intracellular responses, and the first discovered Wnt receptors were the Fz proteins (Wondarz A et al. 1998). The Fz genes constitute a big family which belongs to the G-protein-coupled receptors (GPCR)

superfamily, and they encode seven-pass transmembrane proteins that have high affinity for secreted Wnt ligands (Kikuchi A et al. 2007; He X et al. 2004). The Fz receptors have been found in a variety of animal species, and so far there are 10 Fzs reported in humans (Kikuchi A et al. 2007). The Fz receptors are characterized by the N-terminal cysteine-rich domain (CRD), a conserved extracellular region of 120 amino acids, which is required for Wnt-Fz binding (Clevers H. 2006; Kikuchi A et al. 2007). Knockout mouse studies have shown that some Fzs are essential for embryonic development (van Amerongen R et al. 2008).

1.1.1.3.2 Coreceptors

Low density lipoprotein receptor-related proteins (LRP) constitute a subfamily of LDL receptor family, an ancient family of endocytic receptors (Kikuchi A et al. 2007; He X et al. 2004). The LRP family is constitutively endocytosed from the membrane and recycled back to the cell surface (Lillis AP et al. 2005). Unlike LDL receptor which appears to act solely in lipoprotein metabolism, LRPs seem to have other functions in protease degradation, activation of lysosomal enzymes, cellular entry of viruses and toxins, and neurotransmission as well as vasculature protection by regulating cell migration and integrin activity (Herz J et al. 2001; Lillis AP et al. 2005). Moreover, LRP5/6 or Arrow (*Drosophila* homologue), is a type I single-pass transmembrane protein, which has been shown to be indispensable for Wnt signaling cascade and appears to function as Wnt co-receptors (He X et al. 2004). Unlike Fz receptors which are required for Wnt-β-catenin, Wnt-PCP and Wnt-Ca^{2+} pathways, LRP5/6 is specifically

necessary for canonical Wnt-β-catenin pathway, but not for non-canonical pathways (Kikuchi A et al. 2007; Staal FJ et al. 2008).

1.1.1.3.3 Alternative ligands for Fz/LRP

The finding of alternative ligand for Fz receptors changed the dogma of Wnts exclusively binding to Fzs (Hendrickx M et al. 2008). Besides Wnts, there are other types of factors which activate Fz/LRP receptors and serve as alternative ligands of Wnt signaling. One type is a secreted protein with a cysteine-knot motif called Norrin, which was found in the Norrie disease, an X-linked congenital blindness due to vascular defect in the eye (Hendrickx M et al. 2008). Norrin is a specific and high-affinity ligand for Fz4 and play a critical role in vascular development in the eye and the inner ear (Xu Q et al. 2004). Another type of ligand is secreted R-spondin, which is a family of thrombospondin type I repeat (TSR) domain containing proteins (Clevers H. 2006). Four related proteins have been found in this family: R-spondin 1-4, and all of them structurally feature an N-terminal signal peptide, a CRD with 2 furin like domains, a thrombospondin type1 domain, and a C-terminal region with positively charged amino acids (Hendrickx M et al. 2008).

In addition, some factors bind LRP5/6, serving as antagonists for Wnt signaling. One family called Dickkopf (Dkk) which comprises four members (Dkk1-4) and a unique Dkk-3 related protein named Soggy (Sgy), contain two CRDs separated by a linker region (Kawano Y et al. 2003). Dkk1 and 2 have been demonstrated to be specifically bound to LRP6 with high affinity (Mao B et al. 2001). Moreover, another similar secreted ligand called Wise, consisting of 206 amino acids with a cysteine knot-like domain,

shares 38% identity with SOST, and the mutation of SOST gene resulting in truncated product sclerostin has been found responsible for sclerosteosis (a severe and rare bone disease characterized by a progressive craniotubular hyperostosis) (Itasaki N et al. 2003; Semënov M et al. 2005; Kim CA et al. 2008), and both Wise and SOST have been shown to bind LRP5/6 to modulate Wnt pathway (Ellies DL et al. 2006).

1.1.1.3.4 Alternative receptors or coreceptors for Wnt proteins

The discovery of alternative receptors for Wnt proteins confirmed that Wnts are not exclusively binding to Fzs, but they also interact with some receptor tyrosine kinases (RTKs). Ryk (related to tyrosine kinase), a newly identified receptor for Wnt, belongs to the atypical receptor tyrosine kinase family (Lu W et al. 2004). The Ryk family is composed of a single Ryk gene in mammals and also in C. elegance as well as three Ryk genes in Drosophila (Bejsovec A. 2005). Ryk contains extracellular Wnt inhibitory factor (WIF) domain (functionally similar to CRD of Fz receptors), an intracellular PDZ-binding domain and a tyrosine kinase domain (Kikuchi A et al. 2007). Unlike regular RTKs, Ryk family bears unusual kinase domain motifs with mutations in conserved tyrosine kinase residues, resulting in a lack of catalytic activity (Hovens CM et al. 1992; Yoshikawa S et al. 2001; Yoshikawa S et al. 2003). Derailed, the Drosophila homolog of Ryk, was shown to be a receptor for Wnt5 and essential for Wnt5-mediated Drosophila axon guidance (Yoshikawa S et al. 2003). Mammalian Ryk interacts directly with Wnt1 or 3a via its extracellular WIF domain, and is required for the activity of Wnt-1 or -3a (Lu W et al. 2004). The extracellular domain of Ryk forms a ternary complex with Fz8 and Wnt, and the intracellular domain of Ryk interacts with Dsh, which is required for

the TCF activation induced by Wnt3a, suggesting Ryk may be involved in Wnt canonical signaling (Lu W et al. 2004). Another example is receptor tyrosine kinase-like orphan receptor (Ror) proteins, which also constitute a conserved family of tyrosine kinase receptors. Ror receptors are composed of two structurally related proteins, Ror1 and Ror2 (Green JL et al. 2008). Ror-family RTKs feature an extracellular Fz-like CRD, a membrane proximal kringle (KR) domain, and cytoplasmic tyrosine kinase domains and proline-rich domain (Forrester WC et al. 1999; Green JL et al. 2008). Ror2 was shown to interact with Wnt5a its CRD (Oishi I et al. 2003). Unlike Ryk, Ror2 has been recently demonstrated to require tyrosine kinase activity to mediate the inhibitory canonical activity by Wnt5a (Mikels A et al. 2009).

1.1.1.4 Wnt signaling

So far, at least three different Wnt signaling pathways have been described: the canonical Wnt pathway (β-catenin dependent) for cell fate determination, and two non-canonical Wnt pathways (β-catenin independent) including the planar cell polarity (PCP) pathway for tissue polarity and the Wnt-Ca^{2+} pathway for cell movement (Staal FJ et al. 2008 and Katoh M et al. 2007).

1.1.1.4.1 Canonical β-catenin pathway

1.1.1.4.1.1 Overview

In the absence of Wnt ligands, the destruction complex of β-catenin is formed, where Axin, the scaffold protein, interacts with all other components like tumor

suppressor adenomatours polyposis coli (APC), β-catenin and two kinases, glycogen synthase kinase (GSK) 3α/β and casein kinase (CK)1; therefore, GSK and CK1 sequentially phosphorylate β-catenin at Ser/Thr residues (Ser45 by CK1, and then Ser33, 37 and Thr41 by GSK), which is then recognized by β-transducin-repeat-containing protein (βTRCP), a component of an E3 ligase complex, and results in β-catenin ubiquitination and final degradation by 26S proteasome (Figure 1A). Hence, the levels of β-catenin in cytoplasm and nucleus stay low without activating signals due to continuous phosphorylation events, and in the nucleus, the binding of Groucho (a family of nuclear factors that lack DNA-binding activity but interact with transcription factors resulting in transcriptional repression) to transcription factors T cell factor (TCF) /lymphoid enhancer factor (LEF) leads to the inhibition of Wnt-β-catenin signaling target gene transcription (Clevers H. 2006; Staal FJ et al. 2008; Buscarlet M et al. 2007).

In contrast, in the presence of Wnt ligands, the Fz receptor/LRP coreceptor complex activates the canonical signaling pathway (Figure 1B). The formation of Fz/LRP complex through Dvl (mammalian homologue of Drosophila Dishevelled) facilitates the phosphorylation in the cytoplasmic tail of LRP5/6 by CK1 and GSK3, allowing the docking of Axin to the LRP phosphorylation sites. Hence, the recruitment of Axin to the plasma membrane prevents the formation of the destruction complex of β-catenin, and results in the stabilization of β-catenin. The stabilized β-catenin then translocates to the nucleus and physically displaces Groucho from TCF/LEF and promotes the expression of Wnt-β-catenin signaling target genes including *cMyc* and *Cyclin D1* (Clevers H. 2006 and Staal FJ et al. 2008).

1.1.1.4.1.2 Glycogen synthase kinase (GSK) 3β and Akt

GSK3β is a Ser/Thr protein kinase, initially described as a key enzyme in glycogen metabolism, but now known to regulate a diverse array of cell functions, including development, protein translation, cell cycle, and cytoskeleton dynamics (Cohen P et al. 2001; Hardt SE et al. 2002). GSK3β has also been shown to promote cell apoptosis in neuronal cells (Li M et al. 2000), and to activate proinflammatory dendritic cells (Rodionova E et al. 2007). Direct phosphorylation of a tyrosine residue (Y216) in the catalytic domain is associated with an increase of its kinase activity, whereas direct phosphorylation of N-terminal Ser9 is associated with the inhibition of GSK3β activity (Forde JE et al. 2007). Lithium, a mood stabilizer, not only competitively inhibits Mg^{2+} leading to the inhibition of Mg^{2+}-ATP-dependent catalytic activity of GSK3β, but also increases Ser9 phosphorylation of GSK3β, resulting in inhibited GSK3β activity (Wada A et al. 2009), and thereby activating Wnt/β-catenin signaling through stabilizing β-catenin (Rao AS et al. 2005). In the resting cells, GSK3β is constituively active in the absence of the extracellular signal. However, in growth factor signaling, the activation of protein kinase B (PKB)/ Akt leads to the phosphorylation of Ser9 and thereby the inhibition of GSK3β (Pearl LH et al. 2002).

PI3K-Akt pathway can be activated by a variety of extracellular signals, and have been involved in cell proliferation, survival and protein synthesis, and tumor growth. The activation of Akt can inhibit apoptosis through activating pro-survival factors like NF-κB and inactivating pro-apoptotic factors such as Bad (a Bcl-2 family member), FOXOs, caspase-9, as well as GSK3β (Jiang BH et al. 2008; Dillon RL et al. 2007). 3T3-L1 cells

expressing Wnt-1 inhibited cell apoptosis by inducing expression and secretion of IGFs that activated Akt signaling, and inhibited PI3K partially blocked the effect of Wnt-1 on cell apoptosis (Longo KA et al. 2002). Almedida M et al reported that Wnt5a required PI3K/Akt to protect pre-osteoblastic cells from serum withdrawl-induced apoptosis (Almedida M et al. 2005). Purified Wnt3a also induced phosphoylation and activation of Akt as well as the phosphorylation of GSK3β at Ser9 to enhance Wnt/β-catenin signaling in vitro, which was reversed by sFRP-4 (Constantinou T et al. 2008). Also, during DMSO-induced cardiomyocyte differentiation of P19CL6 cells, Akt inhibition abrogated Wnt-3a or-8a-induced canonical Wnt/ β-catenin activity, indicating that PI3K/Akt pathway maintains canonical Wnt cascade during early caridomyogensis (Naito AT et al. 2005). All these studies support that Akt signaling plays a critical role in Wnt cascade.

1.1.1.4.1.3 β-catenin

One of the substrates of GSK3β, β-catenin, display distinct functions in specific intracellular pools: the membrane bound pool of β-catenin is involved in cell-cell adhesion while the cytosolic and nuclear pool of β-catenin transduces signals in Wnt canonical pathway as well as oxidative stress signaling pathway (Dietrich C et al. 2002; Essers MA et al. 2005). The function and localization of β-catenin depends on the state of the cell: in human keratinocytes, β-catenin is throughout the cell promoting proliferation in subconfluent state, while β-catenin translocates to membrane to promote cell adhesion when cells are confluent (Dietrich C et al. 2002). In endothelial cells, adherens junctions are composed of vascular endothelial cadherin (VE-cadherin), and several protein partners including β-catenin to connect VE-cadherin to cytoskeleton, and the dynamic

opening and closure of adherens junctions regulate endothelial permeability and vascular integrity (Dejana E et al. 2008). In mouse embryos where β-catenin is endothelial cell-specifically inactivated, vessels display an abnormal lumen and frequent hemorrhage, indicating the critical role of β-catenin in vascular patterning and permeability (Cattelino A et al. 2003). The function of β-catenin in Wnt canonical signaling has been described previously. Additionally, Essers MA discovered a functional interaction between FOXOs and β-catenin under oxidative stress, and then proposed that upon Wnt signaling which turns TCF on, or upon insulin signaling that turns FOXOs off, β-catenin prefers interacting with TCF rather than FOXO to promote cell proliferation; in contrast, under oxidative stress conditions where FOXO activity is on, β-catenin preferentially binds to FOXOs to induce apoptosis or quiescence (Essers MA et al. 2005; Bowerman B. 2005).

1.1.1.4.2 Wnt-PCP pathway

Polarity is one of the fundamental properties of many cells, and the tissue polarity, or planar cell polarity (PCP) is the generation of uniform orientation of a population of cells within a single plane, which has been demonstrated in organisms from flies to humans (Fanto M et al. 2004; Montcouguiol M et al. 2006). Drosophila provides numerous striking examples of PCP in its body, such as uniform organization of hairs on the wing, and the ommatidial polarity in the eye (Fanto M et al. 2004). One noncanonical pathway utilizes Fz and Dishvelled (Dvl), as well as many other signaling molecules to regulate the formation of PCP, which has been proved to be crucial for Drosophila morphogenesis and vertebrate gastrulation (Kim GH et al. 2005). In one hand, Dvl is in association with Dishevelled-associated activator of morphogenesis (DAAM) to initiate

Ras homologue gene-family member A (RhoA) – Rho-associated coiled-coil containing protein kinase (ROCK) signaling cascade, which regulates cytoskeletal reorganization; in the other hand, Dvl also activates another small GTPase Rac, both Rac and RhoA can stimulate stress responsive Jun N-terminal kinase (JNK) signaling to regulate cytoskeleton dynamic and cell shape (Montcouguiol M et al. 2006; Staal FJ et al. 2008) (Figure 1.2A). The Wnt-PCP pathway also includes some other components, like protein tyrosine kinase 7 (PTK7, which recruits Dsh), the four-pass transmembrane protein Van Gogh (Vang, core factor in PCP pathway via recruiting Dsh), Drosophila Flamingo/vertebrate Celsr (encoding an atypical proto-cadherine functioning in recruiting Fz and Vang), and Ror2/Ryk receptors seem to be involved in PCP signaling transduction (Montcouguiol M et al. 2006; Katoh M et al. 2007; Shnitsar I et al. 2008; Heisenberg CP et al. 2002; Chen WS et al. 2008).

1.1.1.4.3 Wnt- Ca^{2+} pathway

The other β-catenin-independent pathway is Wnt-Ca^{2+} pathway, which utilizes calcium as a second messenger in this cascade, and has been shown required for body plan specification during development (Kohn AD et al. 2005). Fz receptors, containing seven-pass membrane GPCR structure, appear to function in association with G proteins (Kohn AD et al. 2005; Katanaev VL et al. 2009). When Wnt ligand (e.g. Wnt5a) binds Fz receptor (e.g. Fz2), phospholipase C (PLC) is activated through heterotrimeric GTP binding proteins (e.g. Gβγ subunits) and Dvl protein (Slusarski DC et al. 1997). This in turn causes the cleavage of phosphatidylinositol-4, 5-bisphosphate to inositol trisphophate (IP3) and diacylglycerol (DAG) (Figure 1.2B). IP3 binds to its receptor

localized in the endoplasmic reticulum membrane and renders ER-stored calcium release to cytoplasm, and the increased calcium concentration activates PKC and calcium/calmodulin kinase II (CamKII), which in turn stimulates Nemo-like kinase (NLK) and transcription factor nuclear factor of activated T cells (NFAT), resulting in cell fate regulation and cell movement (Ciani L et al. 2005; Katoh M et al. 2007; Staal FJ et al. 2008).

1.1.1.4.4 Other Wnt signaling pathways

Ryk and Ror proteins are newly identified receptors for Wnt, which has been previously described.

1.1.1.4.5 Dishevelled

Notably, Dishevelled (Dsh) is the protein involved in all the Wnt pathways, and governs a lot of developmental processes of animals ranging from Hydra to humans (Wallingford JB et al. 2005). Dsh protein contains three conserved elements in structure: 1) N- terminal DIX (Dishevelled/Axin) domain featuring a lot of α-helix; 2) central PDZ (PSD-95, DLG, ZO1) domain with a hydrophobic cleft responsible for binding to other proteins; and 3) DEP (Dishevelled, EGL-10, Pleckstrin) domain with a bundle of three α-helices (Wallingford JB et al. 2005). And the unique structure of Dvl proteins provides docking sites for a diverse set of protein kinases, phosphatases, adaptor proteins, G proteins and scaffolds, and as a “toolbox” for Wnt signaling, Dsh involves in different signaling with distinct domains (Malbon CC et al. 2006). In canonical β-catenin pathway, Dvl-associated proteins include Fz1, Axin, CK1, GSK-binding protein/FRAT (preventing

β-catenin phosphorylation), Dapper (promoting Dvl degradation) and IDAX (competing with Axin for binding Dvl); in Wnt-PCP pathway, Dvl interacting proteins include Fz1, Go, DAAM, Vang, Diego (sequestering Dsh to Fz/PCP pathway), Prickle, Rac1; in Wnt-Ca2+ pathway, the interacting partners of Dvl include Fz2, Go/Gt (Wallingford JB et al. 2005; Malbon CC et al. 2006; van Amerongen R et al. 2005; Gao X et al. 2008; Hino S et al. 2001; Wu J et al. 2008). In addition, Wnt3a has been shown to induce Dvl phosphorylation via CKI, and CKI activates Wnt pathway, suggesting that CKI-mediated Dvl phosphorylation might be capable of transducing Wnt cascade (McKay RM et al. 2001).

1.1.1.4.6 The activation/inhibition of Wnt signaling

Some alternative ligands for Fz/LRP receptors and alternative receptors for Wnts can activate Wnt cascade and serve as the agonists of Wnt signaling. Norrin-Fz4 pair activates the canonical Wnt cascade in an LRP5/6-dependent manner (Xu Q et al. 2004). Other ligands like R-spondin and Ryk receptor, also activate canonical β-catenin/TCF pathway (Hendrickx M et al. 2008; Lu W et al. 2004). Moreover, Lithium serves as an activator of canonical β-catenin/TCF signaling through inhibited GSK-3β (Wexler EM et al. 2008).

However, there are some other alternative ligands/receptors inhibiting Wnt cascades and they function as antagonists in Wnt signaling. Soluble Frizzled-related proteins (sFRPs) are the largest family of Wnt antagonists that contain a cysteine-rich domain (CRD) which resembles the ligand-binding domain of Fzs. sFRPs directly bind to both Wnts and Fz receptors, and thus might have dual effect: antagonist of Wnt signaling

but agonist for specific Fz-signaling pathway or Fz-functions, (Clevers H. 2006; Bovolenta P et al. 2008). The inhibitory function of sFRPs may be caused either by interaction and sequestration of Wnts from Fzs or formation of nonfunctional complexes with Fz receptor (Bafico A et al. 1999). But sFRPs was shown to interact with Fz2 and regulate the growth of retinal ganglion cell axons, implying that sFRPs might activate canonical Wnt signaling in some cases (Rodriguez et al. 2005).

Unlike sFRPs, there are some Wnt inhibitors functioning through LRPs. Secreted Dkk-1 blocks activation of the Wnt signaling cascade by interacting with LRP5/6 instead of Wnts *per se*, which in turn, crosslinks LRP6 to Kremens, leading to the formation of a ternary complex including Kremen (Krm), DKK and LRP5/6 and facilitating the internalization of LRP6 from the cell surface, which in turn, inhibits the downstream β-catenin-TCF signaling the internalization of LRP (Hendrickx M et al. 2008; Nakamura T et al. 2008). Krm was originally isolated as a novel type I transmembrane protein containing an extracellular kringle domain which is a homologous triple-disulfide-linked peptide region (Nakamura T et al. 2008). Both Krm1 and Krm2 were shown to be the high affinity receptors for secreted Dkk (Mao B et al. 2002). Another example is SOST/Sclerostin: SOST binds to both LRP5 and LRP6, destroying the formation of Fz-LRP complex in the presence of Wnt1, hence blocking the Wnt1-β-catenin signaling (Hendrickx M et al. 2008). In addition, Ror receptors interacts with Wnt5a via its CRD, and then activates JNK pathway and/or inhibits β-catenin-TCF pathway (Oishi I et al. 2003). Ror also has other ways to signal, such as sequestering Wnt ligands, interacting with Van Gogh to affect cell polarity, forming dimers to promote osteoblast differentiation (Green JL et al. 2008).

However, WISE turned out to activate or inhibit Wnt signaling in a context-dependent manner: Wise activates the Wnt-β-catenin pathway in animal cap; whereas Wise blocks Wnt8-induced secondary axis formation (Itasaki Net al. 2003).

Also, Wnt ligand is not limited to only one pathway, so one question arises that how different Wnt ligands preferentially choose these pathways? The specificity may be achieved by the context of receptors and the availability of coreceptors and/or other signaling molecules (Kohn AD et al. 2005). For example: Wnt5a interacts with Fz2 preferentially activating Wnt-Ca^{2+} cascade (Slusarski DC et al. 1997); in association with LRP5, Wnt5a binds Fz4 to stimulate Wnt- β-catenin pathway (Verkaar F et al. 2009); however, in the presence of coreceptors like collagen triple helix repeat containing 1 (Cthrc1) and Ror2, Wnt5a bound with Fz5 or 6 can initiate Wnt-PCP signaling pathway (Yamamoto S et al. 2008). And Table 1 shows more examples of different Wnt/Fz/coreceptor interaction which specifically induces distinct downstream signaling cascade.

1.1.2 Functions of Wnts and Wnt signaling

Together with fibroblast growth factor (FGF) pathway, Notch signaling, transforming growth factor (TGF) β pathway and Hedgehog signaling, Wnt signaling is recognized as one of the handful powerful pathways that control basic development and organism homeostasis. (Cadigan KM et al. 1997. Wang J et al. 2007. Clevers H. 2006 and Staal FJ et al. 2008)

Wnts and Wnt signaling are involved in virtually every aspect of embryogenesis and organogenesis, including gastrulation, implantation, the patterning of brain and spin cord, heart formation, hematopoiesis, and the development of liver, limb, kidney, lung, skin and gastrointestinal tract. In addition, the non-canonical Wnt pathways such as Wnt-PCP pathway and Wnt-Ca^{2+} pathway also play a key role in cell polarity and cell movement, leading to the regulation of tissue polarity establishment and morphogenesis by Wnts (Wang J et al. 2007). Knockout mouse models have been proved to be a powerful tool to investigate the role of Wnt signal transduction in mammalian patterning, physiological conditions and disease development (van Amerongen R et al. 2006). Table1 summarizes the roles of individual Wnt in embryogenic development and the phenotypes in available knockout mice. Also, Wnts and Wnt signaling also regulate self-renewal and repair processes in numerous adult tissues, such as gut, hair follicle, skin, and bone (Clevers H. 2006).

The roles of Wnts and Wnt signaling in development result from their function in controlling cell fate, including cell proliferation or self-renewal, differentiation, apoptosis and senescence, which has been extrapolated to many different systems, such as intestine, hematopoiesis, CNS, skin, liver, adipogenesis, and bone (Hayward P et al. 2008).

Wnt/β-catenin signaling has been shown the dominant force in controlling cell fate along the crypt-villus axis in small intestine. In neonatal mice lacking TCF4, the differentiated villus epithelium appears unaffected, but crypt progenitor compartment is entirely absent, indicating physiological Wnt signaling maintains the crypt progenitor compartment (Radtke F et al. 2005). Also, Wnt3a treatment can promote cell

proliferation, reduce apoptosis, and inhibit osteogenic differentiation of multipotential adult mesenchymal stem cells (MSCs) (Boland GM et al. 2004). For midbrain dopaminergic development, Wnt3a can increase the proliferation of precursor cells, whereas purified Wnt5a induces immature primary precursors into dopaminergic neurons (Schulte G et al. 2005). Overexpression of constitutively active β-catenin expands the pool of hematopoietic stem cells (HSCs) in vitro and maintains their immature phenotype (Reya T et al. 2003).

Wnt signaling is also a critical modulator in adipocyte differentiation. NIH3T3-L1 preadipocytes treated with lithium or infected with Wnt1 or stabilized β-catenin showed abolished differentiation to adipocytes, which was rescued by PPARγ or C/EBPα infection; canonical Wnt10b had similar inhibitory effect on adipogenesis *in vitro* (Ross SE et al. 2000). Christodoulides C et al gave the first evidence of the possible role of Wnt signaling in human early adipogenesis: they found DKK1, a Wnt antagonist, had a transient upregulation in expression during human adipogenesis and ectopic expression of DKK1 inhibited Wnt-β-catenin activation and promoted preadipocyte differentiation (Christodoulides C et al. 2006).

Moreover, Wnts and Wnt signaling are also involved in cell senescence and aging. Wnt/β-catenin signaling was shown to be enhanced in muscle from aged mice and in myogenic progenitors exposed to aged serum; and the cell fate conversion of myogenic progenitor by aging was also mediated by Wnt/β-catenin signaling (Brack AS et al. 2007). Increased Wnt/β-catenin signaling induced cell senescence both in vivo and in vitro, which may be via klotho protein, because klotho is an antagonist of Wnt/β-catenin

signaling, and mice lacking klotho displayed accelerated aging phenotypes (Liu H et al. 2007). Additionally, Lithium, the inhibitor of GSK-3β was found to induce endothelial cell senescence via stabilizing p53 and upregulating the expression of matrix metalloproteinase-1 (Mao CD et al. 2001; Struewing IT et al. 2009).

Besides, Wnts and Wnt signaling are also implicated in cell polarity and orientation. The uniform orientation of mechanosensory hair cells in the inner ear is a good example for vertebrate cell polarity (Montcouquiol M et al. 2006). In vivo study showed that mutation in Vangl2 led to significant defects in the polarization of stereociliary bundles in mouse cochlea due to the disruptions in the orientation of movement of the kinocilium in hair cells, indicating Vangl2 is a core Wnt-PCP factor with a crucial role in the generation of uniform bundle orientation (Montcouquiol M et al. 2003). In the presence of a chemokine gradient, Wnt5a increased the percentage of cells with the Wnt-mediated receptor-actin-myosin polarity (W-RAMP) structure which was accumulated asymmetricaly at the cell periphery, triggering membrane contractility and nuclear movement in the direction of membrane retraction; and this effect by Wnt5a was found via reciprocal interaction with actin and myosin IIB, as well as the involvement of small GTPase Rab4 and RhoB (Witze ES et al. 2008). Also, Wnt11 was demonstrated to play a key role in the oriented elongation of the myocytes during early chick myogenesis through JNK, because Wnt11 SiRNA showed a failed or mis-oriented enlongation, and ectopic Wnt11 significantly promoted myocyte orientation (Gros J et al. 2009).

In addition, Wnts and Wnt signaling are also important for cell migration. Wnt5a was shown upregulated in T cells upon the treatment of CXC chemokine ligand (CXCL)-

12, and Wnt5a potentiated CXCL-12-stimulated T cell migration through PKC activation (Ghosh MC et al. 2009). Wnt3a, a canonical Wnt/β-catenin signaling activator, not only induced HUVEC proliferation, but it also induced HUVEC migration, accompanied with the upregulation of the target genes such as c-myc, Tie-2 and GJA-1 (Samarzija I et al. 2009). Moreover, intestinal epithelial cells incubated in conditioned media from Wnt11-secreting cells were shown to be stimulated in cell proliferation and cell migration, as well as increased activity of PKC and CAMKII, with abnormal distribution of E-cadherin (Ouko L et al. 2004).

The role of Wnts in cell apoptosis will be discussed in detail later.

1.1.3 Wnt and diseases

Due to the crucial roles in organism development and postnatal homeostasis, Wnts and Wnt signaling are highly related to diseases: germline mutations in the Wnt pathway lead to certain hereditary diseases, and somatic mutations are associated with cancer and other human disorders (Clevers H. 2006).

1.1.3.1 Cancer

Normally, Wnt/β-catenin signaling tightly controls the proliferation and renewal of stem cells and progenitors during the development and regeneration of tissues, like intestinal crypt, hair follicles, and hematopoietic systems. Nonetheless, deregulated Wnt signaling can cause aberrant expansion of the stem cell pool and constitutive activation of

stem cell function on progenitor cells, promoting tumor formation in these tissues, including colon cancer, hair follicle tumors and leukemia (Reya T et al. 2005. Clevers H. 2006). It is noteworthy that without the involvement of some key components of Wnt signaling, Wnt ligands alone are rarely related to the activation of Wnt pathway during carcinogenesis (Giles RH et al. 2003).

1.1.3.1.1 Colon cancer

Physiological Wnt signaling appears to be indispensible for the establishment of crypt stem/progenitor compartment in small intestine epithelium; yet, when APC is mutated, the stablized β-catenin activates crypt stem/progenitor cell proliferation and causes tumor formation (Clevers H. 2006). APC was first identified as a tumor suppressor in human colon cancer, because the germline mutation of APC (mainly truncation) is responsible for a hereditary cancer called Familiar Adenomatous Polyposis (FAP), which progresses to colorectal adenomas, and malignant adenocarcinomas, as well as tumors elsewhere like gastrointestinal tract, brain and thyroid (Polakis P et al. 1997). Not only in FAP, a rare form of cancer, APC mutations are also found in approximately 85% of somatic colorectal cancer, which is the most common human neoplasms (Bienz M et al. 2000 and Giles RH et al. 2003). However, recent studies revealed that loss of APC alone is not sufficient to induce tumor formation in zebrafish, but contribute to adenoma initiation as the first step, while KRAS-mediated nuclear localization of β-catenin is required for intestinal cell proliferation as the second step in promoting adenoma progression to carcinomas (Phelps RA et al. 2009). Besides Wnt signaling components, Wnt2 was shown upregulated in 74 cases out of 120 colorectal cancers, and Wnt2 may be associated with the development rather than progression of

colorectal cancers (Park JK et al. 2009). Moreover, Wnt2 was also shown to be overexpressed in other digestive neoplasms like gastric cancer and esophageal carcinomas (Vider BZ et al. 1996). Wnt antagonists were also found to be associated with colorectal cancer. For sFRP-1, hypermethylation (less transcription) and down-regulated mRNA levels were identified significantly more often in tumor samples than normal samples (Caldwell GM et al. 2004). Moreover, DKK-1 was also found hypermethylated in 17% primary colorectal tumor cases, and the CpG island promoter of DKK-1 was selectively hypermethylated in advanced Duke's stages of human colorectal neoplasms (Aguilera O et al. 2006).

1.1.3.1.2 Skin cancer

Normal Wnt cascade in hair follicles activates epidermal stem cells in bulge area, promoting the entry into hair lineages, and cell migration to transit-amplifying germinative matrix, resulting in hair formation (Clevers H. 2006). Both trichofolliculoma and pilomatricoma are tumors with elements of follicular differentiation, and they both contain cells undergoing differentiation along hair lineages from epidermal stem cells that reside in hair follicle (Owens DM et al. 2003). Transgenic mice expressing a stabilized β-catenin form in the epidermis and hair follicles developed trichofolliculoma-like tumors in the mouse skin, and older mice developed pilomatricoma-like lesion (Gat U et al. 1998). This observation was also seen using tamoxifen-induced stabilized β-catenin transgenic mice which developed tumors resembling trichofolliculomas upon continuous activation of β-catenin (Lo Celso C. et al. 2004). In addition, most spontaneous pilomatricomas in humans carry constitutive mutations in β-catenin (Clevers H. 2006).

Besides hair follicle tumor, Wnts are also related to other skin cancers. In a study with 59 primary melanoma cases, cytoplasmic Wnt5a expression is increased with melanoma progression, and the strong Wnt5a expression is a risk factor for metastasis and reduced survival (Da Forno PD et al. 2008). And the increased mobility of melanoma cells by Wnt5a is mediated through the inhibition of metastasis suppressors, such as KISS-1 (a gene encoding kisspeptin which is a peptide ligand of a GPCR called GPR54 to block chemotaxis) , and is associated with an epithelial to mesenchymal transition (EMT) in a PKC-dependent manner (Dissanayake SK et al. 2007).

1.1.3.1.3 Leukemia

Hematopoietic stem cells (HSCs), the best studied stem cells in human body, are controlled by Wnt cascade in their self-renewal and normal growth (Reya T et al. 2005). Moreover, like stem/progenitor cells in crypt or hair follicles, HSCs can also be the target of mutational Wnt/β-catenin signaling to cause leukemia (Clevers H. 2006). Wnt5a null mice showed 35% increase in the proportion of B lineage cells and 55% increase of B cell number, and Wnt5a heterozygous mice developed B cell lymphomas and chronic myeloid leukemia, indicating Wnt5a is able to suppress hematopoietic malignancies (Liang H et al. 2003). Jamieson CH and coworkers reported that the granulocyte-macrophage progenitors from patients with chronic myelogenous leukemia (CML) displayed striking increase of nuclear β-catenin and LEF/TCF activity compared to normal cells (Jamieson CH et al. 2004). Wnt signaling is also related to acute myeloid leukemia (AML) since AML fusion proteins induced TCF/LEF target gene transcription *in vitro* (Müller-Tidow C et al. 2004). Moreover, some evidences support that abnormal Wnt signaling is associated with acute lymphoblastic leukemia (ALL); e.g. conditional

activation of β-catenin by deleting exon3 in mice developed aggressive thymic lymphomas, and this process was not Notch signaling required, which is the first report demonstrating that aberrant Wnt/β-catenin cascade alone in the thymus can lead to leukemia (Guo Z et al. 2007). Similarly, mice transplanted with bone marrow cells expressing constitutively active mutant of LEF-1 developed B lymphoblastic leukemia and AML at 12 months, proving the crucial role of LEF-1 activity in normal lymphopoiesis (Petropoulos K et al. 2008). Besides, DKK1 was found upregulated in the serum from patients with diagnosed myeloma (Tian E et al. 2003).

1.1.3.1.4 Other cancers

Wnt-1 was found as an oncogene activated by integration with tumor virus MMTV to induce murine breast cancer (Nusse R et al. 1982). Moreover, Axin mutation has been found in hepatocellular carcinoma (HCC) cell lines and primary HCCs (Satoh S et al. 2000), and mice with deletion of APC in liver developed HCC (Colnot S et al. 2004), demonstrating the association of activated Wnt-β-catenin signaling with HCC. Prostate cancer samples were also shown to carry β-catenin mutations (Gersterin AV et al. 2002). Additionally, in Wilms Tumor (WT) which is a kidney cancer, genetic abnormalities were identified, such as mutations in β-catenin and WT gene on the X-chrmosome (WTX), a tumor suppressor preventing β-catenin from translocating to the nucleus (Nusse R. 2007).

The effect of Wnt5a in tumor development and progression is dependent on the cancer types: as discussed previously, Wnt5a promotes metastasis in melanoma; however, Wnt5a transfection can suppress cell proliferation, migration, invasiveness and

clonogenicity in thyroid tumor cell lines, which is associated with the inhibition of canonical β-catenin signaling, implying that Wnt5a has tumor suppressor activity in thyroid carcinoma, which is consistent with the finding in B cell lymphoma (Kremenevskaja N et al. 2005; Liang H et al. 2003). Furthermore, the up-regulated expression of Wnt5a is related to the early stages of lip carcinoma (Xavier FC et al. 2009).

1.1.3.2 Alzheimer's Disease (AD)

Wnt signaling plays an important role not only in the early phases of the nervous system development during embryogenesis, but also in self-renewal processes, including maintenance of adult neural stem cells, refinement of neuronal circuits (axon, dendrites, synapse) and adult hippocampal neurogenesis (Toledo EM et al. 2008; Michaelidis TM et al. 2008). AD is known as a neurodegenerative disease characterized by diffuse loss of neurons, protein deposits including neurofibrillary tangoes and amyloid plaques (De Ferrari GV et al. 2000). The activation of Wnt/β-catenin signaling has shown neuroprotective roles in AD models. Wnt3a prevented Aβ-induced neurotoxicity in hippocampal neurons (Alvarez AR et al. 2004). Therapeutic concentrations of lithium (GSK3β inhibitor) treatment dramatically reduced the production of Aβ peptides *in vitro* and *in vivo*, which was achieved by interfering with the cleavage of amyloid precursor protein (APP) (Phiel CJ et al. 2003). Furthermore, De Ferrari GV and coworkers reported that *LRP6* single nucleotide polymorphisms (SNPs) were associated with late-onset AD in both muticenter case-control and family-based series, and Ile1062 --> Val substitution

which reduced β-catenin activation was also found, showing the involvement of Wnt signaling components in AD (De Ferrari GV et al. 2007). Furthermore, presenilin 1 (PS1) is a causative gene product of familial AD, and GSK-3β-mediated phosphorylation of PS1 inhibited its interaction with N-cadherin, resulting in impaired activation of cell-cell contact-induced Akt survival pathway, suggesting the possible involvement in reduced neuronal viability and synaptic plasticity (Uemura K et al. 2007). In the other hand, GSK-3β-mediated PS1 phosphorylation induced a structural change of PS1, which in turn reduced PS1-β-catenin interaction and led to decreased phosphorylation and degradation of β-catenin, resulting in enhanced β-catenin nuclear signaling (Prager K et al. 2007)

1.1.3.3 Metabolic syndrome

Canonical Wnt signaling activity was observed in pancreatic β cell lines and mature islets (Welters HJ et al. 2008), and some evidences support that Wnt/β-catenin signaling may regulate β cell function. Wnt10b transgenic mice showed improved glucose tolerance and insulin sensitivity (Longo KA. 2004). Additionally, LRP5 deficient mice displayed impaired glucose-induced insulin secretion under chow diet, which was restored by the infection with adenovirus encoding LRP5; also LRP5+/+ islets had increased insulin secretion pretreated with Wnt3a or 5a conditioned media (Fujino T et al. 2003).

Wnt5b was shown to be associated with type II diabetes in Japanese population (Kanazawa A et al. 2004). And another studies demonstrated the association of transcription factor 7 like – 2 (TCF7L2, previously TCF4) polymorphism with the risk in

type 2 diabetes in three Caucasian cohorts. In Grant SF's study, a microsatellite, DG10S478, located in the intron 3 of TCF7L2, was found to be related to type 2 diabetes, and the homozygous carriers of at-risk allele, a DG10S478 composite had around 2.4 times more risk of type 2 diabetes than noncarriers (Grant SF et al. 2006). Then a similar study was done in Amish population, and the data further indicated that TCF7L2 variants likely influence insulin sensitivity (Damcott CM et al. 2006).

For in vivo studies, FABP4-Wnt10b transgenic mice showed less adipose tissue and more resistance to diet-induced obesity (Longo KA. 2004) compared to wildtype mice; and under ob/ob or agouti background, FABP4-Wnt10b transgenic mice still displayed reduced body weight, tissue weight and white adipose tissue (Wright WS et al. 2007). All these studies indicate that Wnt signaling inhibit obesity formation in mice.

1.1.3.4 Cardiovascular diseases

Wnt cascade is of significance in normal heart development, and abnormal Wnt signaling has been shown to be associated with several heart diseases (van de Schans VA et al. 2008). Using immunohistochemical analysis, coincident expression of both Wnt5a and TLR-4 was found in murine and human atherosclerotic lesions (Christman MA et al. 2008). Conditional deletion of β-catenin in mouse cardiomyocytes resulted in attenuation of cardiac hypertrophy induced by pressure, suggesting β-catenin is the regulator of cardiomyocyte growth (Chen X et al. 2006). Mani A et al characterized a family with extraordinary prevalence of early coronary artery disease (CAD), also featuring metabolic syndromes (hyperglycemia, hyperlipidemia, and hypertension). A missense

mutation (R611C) in *LRP6* gene was then identified in most family members through genotyping studies, linking Wnt signaling to the development of CAD (Mani A et al. 2007).

1.1.3.5 Bone diseases

Bone mass is regulated by the balance between osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells), and osteoporosis comes from the imbalance between bone resorption and bone formation. Wnt/β-catenin signaling promotes osteoblasts expansion and function, playing a particularly important role in bone biology (Westendoff JJ et al. 2004; Krishnan V et al. 2006). Mutation of human LRP5 was found to cause an autosomal recessive disorder osteoporosis-pseudoglioma syndrome (OPPG), featuring low bone mineral density (Gong Y et al. 2001). Also, deletion of sFRP-1 led to increased trabecular bone formation as well as decreased apoptotic osteoblasts and osteocytes in adult mice, and to stimulate osteoclastogenesis *in vitro* (Bodine PV et al. 2004). Similarly, transgenic mice with expression of Wnt10b in marrow also showed increased bone mass and strength, more resistance to bone loss due to aging or estrogen deficiency, further suggesting that the involvement of Wnt/β-catenin signaling in bone mass, and the underlying mechanism could be that Wnt10b inhibits PPARγ –mediated adipogenic program and shifts the cell fate of bipotential mesenchymal precursors to the osteoblast lineage (Bennett CN et al. 2005).

1.1.4 Wnt and vasculature

1.1.4.1 Wnt and vascular biology

Genetic deletions or mutations of Wnt or Wnts signaling components provide the evidences that Wnt is required for vascular development and function. Wnt2 deficient mice died perinatally due to the severe placental defects with visible hematomas, abnormal large maternal blood pools, and reduced number of fetal capillaries, suggesting Wnt2 is indispensible for the proper vascularization of mouse placenta development (Monkley SJ et al. 1996). Wnt7b is another example: germline knockout of Wnt7b in mice led to hemorrhages surrounding the pulmonary vessels caused by defects in the differentiation (aberrant expression of smooth muscle α-actin) and maintenance of vascular smooth muscle cells (more apoptosis of vascular smooth muscle cells) (Shu W et al. 2002). Another study showed that Wnt7b mutant mice failed to drive hyaloids vessel regression in the developing eye, which is an indication of correlation between Wnt7b and cell apoptosis required for vasculature (Lobov IB et al. 2005). Moreover, Fz5 conventional knockout mice displayed defective yolk sac and placental vasculature (Ishikawa T. 2001), further suggesting the regulatory role of Wnt signaling in vessel development. More recently, Stenman JM et al developed Wnt7a/7b double mutant mice and showed that these mice had a severe CNS-specific hemorrhaging phenotype due to endothelial abnormalities, strongly indicating that Wnt7a/7b and canonical Wnt signaling directly target vascular endothelium to promote vasculature formation in the central nervous system in mouse embryos and to initiate the development of blood-brain barrier (Stenman JM et al. 2008).

1.1.4.2 Wnts and vascular diseases

The discovery of association between Wnt/β-catenin signaling and human vascular disease was with Fz4 mutations in familial exudative vitreoretinopathy (FEVR), a hereditary ocular disorder characterized by excessive vascular branching due to failure of vessel regression, retina detachments and leaky vasculature (Miyakubo H et al. 1982; Robitaille J et al. 2002; Masckauchán TN et al. 2006). Another type of disease related to Fz4 is Norrie disease, which is a sex-linked, congenital disorder with fibrous and vascular changes of the retina causing visual impairment (Zerlin M et al. 2008). Xu Q et al also reported that Fz4 null mice displayed intraretinal vascular defects in adulthood (Xu Q et al. 2004), indicating the critical role of Fz4 in retina vasculature. Furthermore, unlike Fz5 whole-body knockout mice described before, Fz5 retina-specific knockout mice developed phenotypes similar to human eye disease persistent hyperplastic primary vitreous (PHPV), characterized by accumulation of retrolental tissue, failure of vessel regression, and abnormal retina morphogenesis (Zhang J et al. 2008), also proving that abnormalities in Wnts and Wnt//β-catenin signaling are responsible for vascular pathogenesis.

In zebrafish, Wnt13 (Wnt2bb) was detected in posterior lateral plate mesoderm (LPM) during somitogenesis, predominantly in cells who are committed to endothelial and hematopoietic lineages, and expressed in the developing vasculature, endocardium, atrioventricular valve in the clater stages (Ober EA et al. 2006). Additionally, all three isoforms of Wnt13 have been found in differentiated endothelial cells, (Struewing IT et al. 2006), and the differential function of Wnt13 isoforms in endothelial cells will be discussed in following chapters.

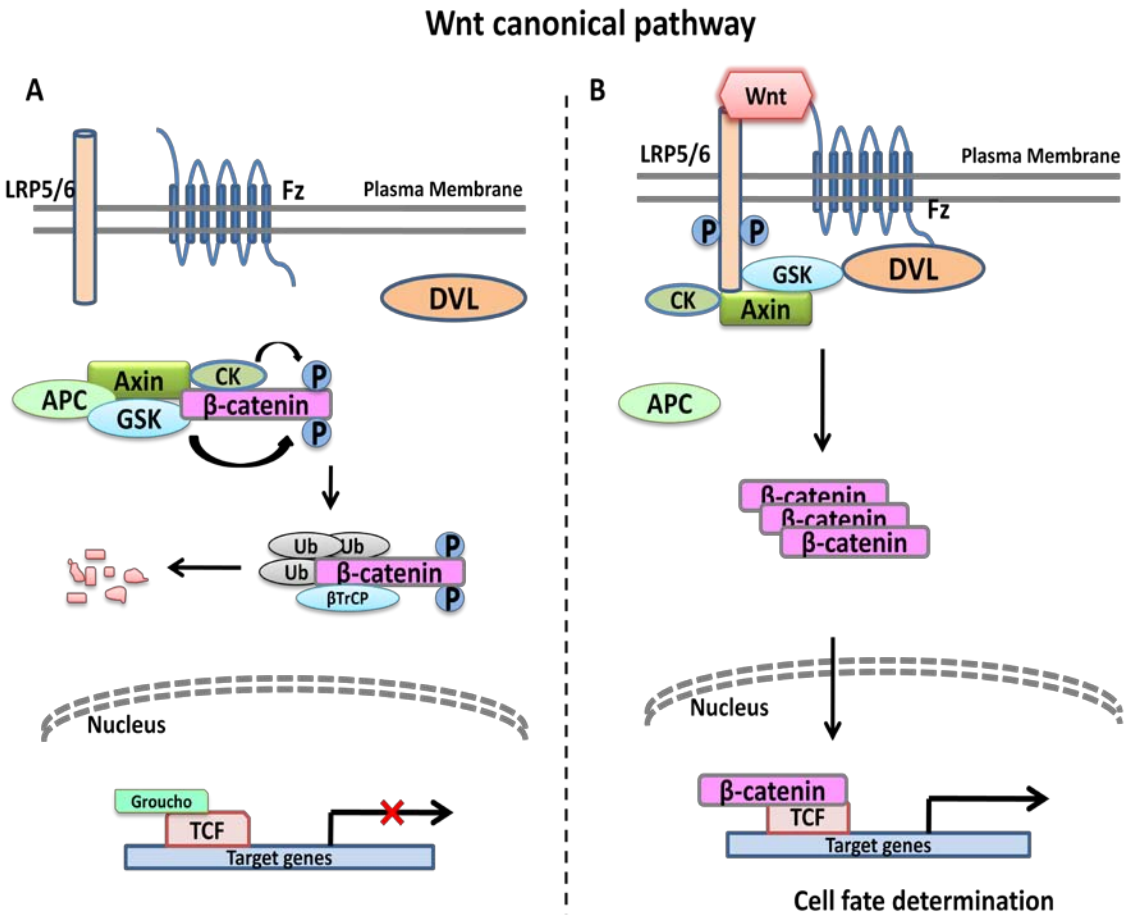


Figure 1.1.1 Wnt/β-catenin canonical pathways. A) In the absence of Wnts (e.g. Wnt1, 3a), the scaffold Axin assembles the destruction complex of β-catenin which contains APC, GSK3, CK1 and β-catenin. In this complex, β-catenin is sequentially phosphorylated by CK1 and GSK3, and then recognized by β-TrCP, a component of E3 ligase which conjugates β-catenin with ubiquitin, resulting in the degradation of β-catenin by proteasome. In the nucleus, the binding of transcription repressor Groucho to transcription factors T cell factor (TCF) /lymphoid enhancer factor (LEF) leads to the inhibition of Wnt-β-catenin signaling target gene transcription. B) In the presence of Wnts (e.g. Wnt1, 3a), Axin is recruited to cell surface through LRP5/6 phosphorylation.

Without the formation of destructive complex, the phosphorylation and degradation of β-catenin is thereby inhibited, so β-catenin is stabilized and accumulated in the cytosol, and then translocates to the nucleus to displace Grouncho and activate TCF transcription factor to initiate Wnt target gene transcription (He X et al. 2004).

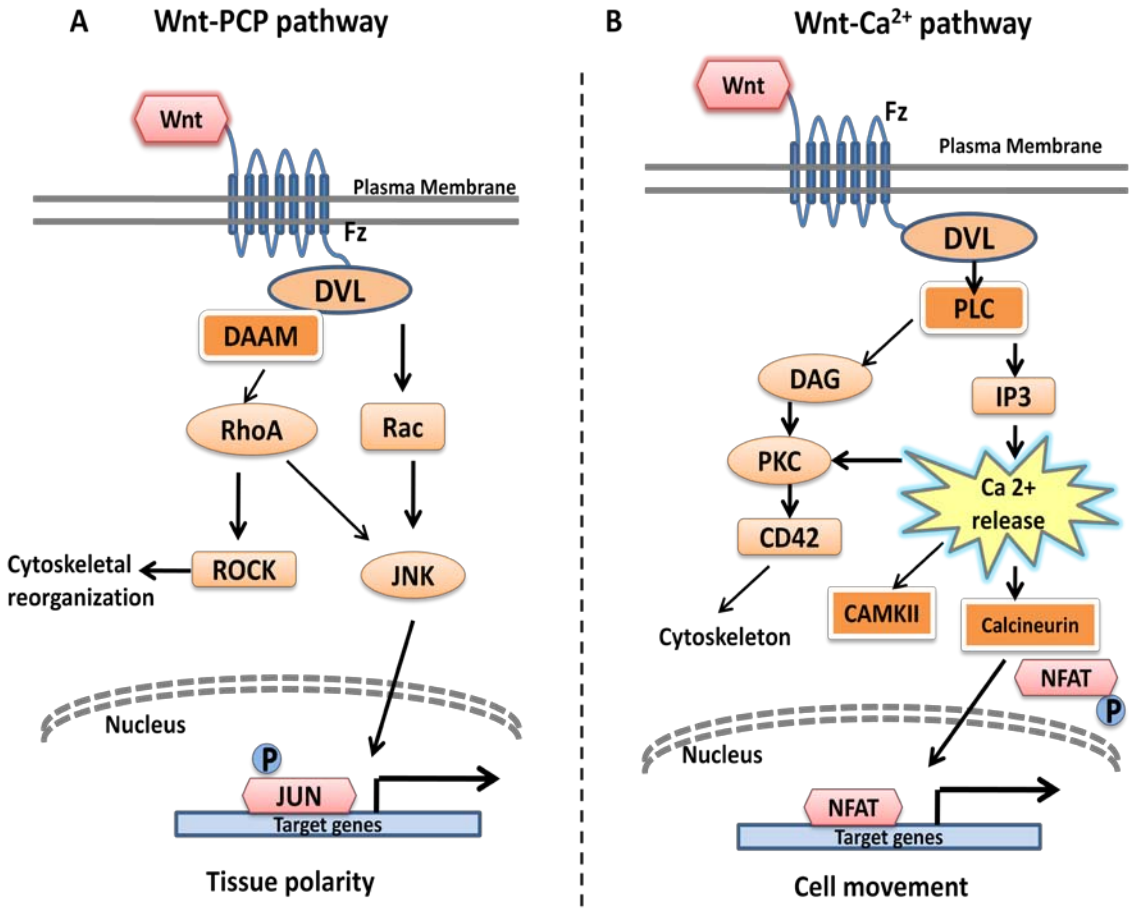


Figure 1.1.2 Noncanonical Wnt pathways. A) Wnt-PCP pathway activates RhoA and Rac through Dvl and DAAM. Subsequently ROCK and JNK signaling are initiated to regulate cytoskeletal remodeling, cell adhesion and mobility. B) Wnt-Ca^{2+} pathway is mediated via heterotrimeric G protein and Dvl, which in turn activates PLC to produce IP3 and DAG, leading to Ca^{2+} release from ER and the activation of PKC, CAMKII, and phosphatase Calcineurin, leading to the regulation of cell fate and the change of cell movement (Staal FJ et al. 2008).

Table 1 The receptors/coreceptors, signaling pathways, phenotypes of knockout mice and other features of individual Wnts

	Bound receptors (coreceptors) and signaling	**Secreted**	**Isoforms**	**Phenotypes of Knockout mice**
Wnt1 (Wg)	Fz1, 2, 5 (LRP6): canonical/β-catenin (Cong F et al.2004) Fz9 (Wang YK et al. 1997) Fz4, 7, 8 (Bhabot P et al.1996)	Yes (Smolich BD et al. 1993)	Not reported yet	Prenatal lethality or antaxia if survival (deficiency in midbrain and cerebellum) (Thomas KR et al. 1990)
Wnt2	Fz1, 4, 5, 9: canonical/β-catenin (Gazit A et al. 1999; Ishikawa T et al. 2001; Karasawa T et al. 2002; Klein D et al. 2008)	Yes (Klein D et al. 2008)	3 different isoforms: variant 1 and variant 2 differ in N-terminus, while variant 3 only contains partial mRNA with exon1 and 2 (Genebank)	Perinatal lethality or reduced birthweight due to placental defects with visible hematomas, abnormal large maternal blood pools, and reduced number of fetal capillaries (Monkley SJ et al. 1996); severe lung hypoplasia with dilated vascular endothelial plexus (Goss AM et al. 2009)
Wnt3	Fz1, 7: canonical/β-catenin (Gazit A et al. 1999; Kim M et al. 2008)	Yes (Kobune M et al. 2007)	Not reported yet	Embryonic lethality with no primitive streak formation (sign of anterior-posterior axis) before gastrulation (Liu P et al. 1999)

Table 1 (continued)

	Bound receptors (coreceptors) and signaling	Secreted	Isoforms	Phenotypes of Knockout mice
Wnt3a	Fz1, 2: canonical/β-catenin (Gazit A et al. 1999; Verkaar F et al. 2009) Fz1: Go activation (Katanaev VL et al. 2009) Fz5, 6 [protein collagen triple helix repeat containing 1 (Cthrc1), Ror2]: Wnt-PCP (Yamamoto S et al. 2008) Fz8 (LRP6) (Cong F et al.2004)	Yes(Smolich BD et al. 1993)	Wnt3a variant 1 and Wnt3a variant 2 (chicken): differ in N-terminus (exon 1). Wnt3a variant 2 induces β-catenin activity. (Narita T. 2007)	Embryonic lethality due to axial truncation as well as defective development of dorsal mesoderm and CNS morphogenesis (Takada S et al. 1994)
Wnt4	Fz1, 2: canonical/β-catenin (Harris KE. 2007) Fz6: non-canonical (Lyons JP et al. 2004) Fz3: (Lyuksyutova AI et al. 2003) (Four jointed, Fj): Wnt-PCP (Lim J et al.2005)	Yes (Smolich BD et al. 1993; Stark K et al. 1994)	Not reported yet	Perinatal lethality with failure in Kidney tubulogenesis (Stark K et al. 1994); Loss of female productive duct formation (Vainio S et al. 1999); Defect in testis differentiation (Jeays-Ward K. 2004)

Table 1 (continued)

	Bound receptors (coreceptors) and signaling	Secreted	Isoforms	Phenotypes of Knockout mice
Wnt5a	Fz1: unknown (Gazit A et al. 1999) Fz1,6,7: Go activation (Katanaev VL et al. 2009) Fz2: Wnt-Ca^{2+} via Gβγ (Slusarski DC et al. 1997) Fz4 (LRP5): canonical/β-catenin (Verkaar F et al. 2009) Fz5: canonical/β-catenin (He X et al. 1997) Fz5, 6 (Cthrc1, Ror2): Wnt-PCP (Yamamoto S et al. 2008)	Yes(Smolich BD et al. 1993)	Not reported yet	Neonatal lethality; defective outgrowth of limbs, face, ears and genitors due to disability of anterior-posterior (A-P) axis extension in embryos (Yamaguchi TP et al. 1999); Abnormal lung morphogenesis (Li C et al. 2002); Impaired pituitary gland shape (Cha KB et al. 2004)
Wnt5b	Fz1: Go activation (Katanaev VL et al. 2009) Fz7: Myogenesis (Linker C et al. 2003) Unknown: Wnt-PCP (Carreira-Barbosa F et al. 2003)	Yes (Smolich BD et al. 1993)	Human Wnt5b variant 1 and 2: differ in 5'-UTR at mRNA level, encoding the same protein (pubmed)	Viable (van Amerongen R et al. 2006)
Wnt6	Fz7: canonical/β-catenin? (Linker C et al. 2005) Fz8 (LRP5): canonical/β-catenin (Semenov MV et al. 2006)	Yes (Smolich BD et al. 1993)	Not reported yet	Viable (van Amerongen R et al. 2006)

Table 1 (continued)

	Bound receptors (coreceptors) and signaling	Secreted	Isoforms	Phenotypes of Knockout mice
Wnt7a	Fz1,6: Go activation (Katanaev VL et al. 2009) Fz5, 10: canonical/β-catenin (Carmon KS et al. 2008b; Kawakami Y et al. 2000) Fz10: Wnt-PCP (Carmon KS et al. 2008a)	Yes (Smolich BD et al. 1993)	Not reported yet	Lack of anterior-posterior and dorsal-vertral axis patterning of limb (Parr BA et al.1995); Female mice were sterile due to posteriorized productive tracts (Miller C et al. 1998); Delayed morphological maturation in cerebellum (Hall AC et al. 2000)
Wnt7b	Fz1, 10 (LRP5): canonical/β-catenin (Wang Z et al. 2005) Fz4: canonical/β-catenin (Lobov IB et al. 2005)	Yes (Smolich BD et al. 1993)	Wnt7b-1 and Wnt7b-2 (mouse and chicken): differ in N-terminus (exon 1) (Rajagopal J et al. 2008 and Fokina VM et al. 2006)	Embryonic lethality due to placental abnormalities (exon 3 deletion) (Parr BA et al. 2001) Perinatal lethality due to respiratory failure (defects in mesenchymal proliferation and vessel development) (exon 1 deletion) (Shu W et al. 2002) Some neonatal lethality while viable mice showing fertile and failure of hyaloids vessel regression (exon 1 deletion) (Lobov IB et al. 2005)

Table 1 (continued)

	Bound receptors (coreceptors) and signaling	Secreted	Isoforms	Phenotypes of Knockout mice
Wnt8a	Fz1: canonical/β-catenin (Yang-Snyder J. 1996) Fz4,5,7 (LRP6): canonical/β-catenin (Holmen SL et al. 2002) Fz2,8 (Hsieh JC. 1999a)	Yes (Hsieh JC. 1999b)	Not reported yet	Viable (van Amerongen R et al. 2006)
Wnt8b	Fz8a: canonical/β-catenin (Kim SH et al. 2002; Cavodeassi F et al. 2005)	Yes (Katoh M et al. 2005)	Not reported yet	Viable (van Amerongen R et al. 2006)
Wnt9a (Wnt14)	Fz4, 7, 9: canonical/β-catenin (Matsumoto K et al. 2008)	Yes (Matsumoto K et al. 2008)	Not reported yet	Neonatal lethality; skeletal defects and chrondroid metaplasia in some joints (Später D et al. 2006)
Wnt9b (Wnt15)	Fz5 (LRP6), Fz8: canonical/β-catenin (Liu C et al. 2008)		Not reported yet	Neonatal lethality with defective urogenital system (Carroll TJ et al. 2005)
Wnt10a			Not reported yet	Unknown
Wnt10b (Wnt12)	Fz8 (LRP5): canonical/β-catenin (Semenov MV et al. 2006) Fz5: canonical/β-catenin (Ishikawa T et al. 2001)	Yes (Ouji Y et al. 2006)	2 isoforms: differ in a central region Exon 3(Wang J et al. 1996)	More myogenic differentiation of myoblasts and increased activation of adipogenic program in muscle injury (Vertino AM et al. 2005); Reduced bone formation rate (Bennett CN et al. 2007)

Table 1 (continued)

	Bound receptors (coreceptors) and signaling	Secreted	Isoforms	Phenotypes of Knockout mice
Wnt11	Fz5 (LRP6): canonical/β-catenin (Holmen SL et al. 2002) Fz5: antagonize canonical/β-catenin (Cavodeassi F et al. 2005) Fz7 (Ryk): Wnt-PCP (Djiane A et al. 2000; Kim GH et al. 2008)	Yes (Kikuchi A et al. 2007)	Not reported yet	Perinatal lethality with smaller size of kidney due to defective ureteric branching patterning (Majumdar A et al. 2003)
Wnt13 (Wnt2b)	Fz7(receptor for Wnt13A): unknown (Struewing IT et al. 2007)	Yes (Wnt13A) (Struewing IT et al. 2006)	Wnt13A, Wnt13B and Wnt13C (human): differ in N-terminus (Katoh M et al.2000 and Struewing IT et al. 2006)	Viable (van Amerongen R et al. 2006) Wnt2/Wnt2b double knockout mice: complete lung agenesis (Goss AM et al. 2009)
Wnt16	Unknow receptors: canonical/β-catenin (Mazieres J et al. 2005)		Wnt16a, Wnt16b (human): differ in N-terminus (exon 1) (Fear MW et al. 2000)	Viable (van Amerongen R et al. 2006)

1.2 Wnt13

1.2.1 General background

The original human Wnt13B (Wnt2b1) cDNA was firstly isolated and characterized from human gastric cancer mRNAs by Katoh M et al in 1996. However, the sequence of the human Wnt13B (Wnt2b1) was different than that of the Wnt13/Wnt2b identified in mouse, chicken and xenopus; and in 2000, the mouse Wnt13/Wnt2b was found equivalent to human Wnt13A/Wnt2b2 (Katoh M. et al. 2000). Besides Wnt13A and Wnt13B, the human Wnt13C was also identified from endothelial cells in 2006 (Struewing IT et al. 2006).

One of the distinguished features of a few Wnts compared to most of Wnts is that they have different isoforms which differ 1) either in N-terminus due to alternative splicing, resulting in distinct subcellular localizations, such as Wnt3a (Narita T et al. 2007), 7b (Fokina VM et al. 2006 and Rajagopal J et al. 2008), 13(Katoh M. et al. 2000; Struewing IT et al. 2006), and 16 (Fear MW et al. 2000); 2) or in the central region, such as Wnt10b. Therefore Wnt13 is one of the few Wnt members with isoforms.

The *Wnt13B* gene is located in human chromosome 1p13 (Katoh M et al. 1996). Due to the difference in the first or second exons, the mRNAs of Wnt13A and Wnt13B differ in the 5'-UTR and N-terminal coding region (Katoh M. et al. 2000). Wnt13B contains exon 1-2, 4-7, which encodes the protein of 372 amino acids including the N-terminal hydrophobic domain; while Wnt13A contains exon 3-7, encoding 391 amino aids without this N-terminal hydrophobic domain (Katoh M. et al. 2000). The Wnt13B mRNA was detected in heart, brain, placenta, lung, prostate, testis, ovary, small intestine,

kidney, and colon (Katoh M et al. 1996). The Wnt13A mRNA was also detected in fetal brain, fetal lung, fetal kidney, caudate nucleus, testis, while Wnt13B expression level was relatively higher in fetal brain and fetal lung than in other tissues (Katoh M. et al. 2000).

Besides the discovery of human Wnt13, the Wnt13 cDNAs were also identified in Xenopus (Wolda, SL et al. 1992), mouse (Zakin LD et al. 1998) , chicken (Jasoni C et al. 1999), zebrafish (Ng JK et al. 2002), rat (Ricken A. et al. 2002) and bovine (Struewing IT et al. 2006; Goodwin AM et al. 2006). Indeed, the Wnt13 found in all these animal species refers to Wnt13A, so it seems that the isoform pattern of Wnt13 only exist in higher class of animals.

Among Wnt family, Wnt2b and Wnt2 are under the same subfamily since the alignment of Wnt sequences shows that Wnt2 has more homology to Wnt2b (72.5% amino acid identity) than other Wnts (Katoh M. et al. 2000), and the evolutionary relationship between Wnt2 and Wnt2b is contributed by duplication of an ancient gene cluster (Katoh M. et al. 2002; Gariock RJ et al. 2007).

1.2.2 Human Wnt13 isoforms

Katoh M. et al firstly described two Wnt13 isoforms derived from the same gene due to alternative splicing. They isolated a novel Wnt13 isoform (Wnt2b2/Wnt13A) in addition to the original Wnt13 isoform (Wnt2b1/Wnt13B). Wnt13A and Wnt13B share the Wnt core domain but differ in the 5’-UTR and N-terminus, resulting in 87.0% amino acid homology between Wnt13A and Wnt13B. Also as shown in Figure 1.2A, Wnt13B

mRNA contains exons 1, 2 and 4-7, whereas Wnt13A contains mRNA contains exons 3-7, and their expression pattern differs too (Katoh M. et al. 2000).

Our laboratory demonstrated that various human cell types, such as differentiated endothelial cells and hematopoietic cells, express three different Wnt13 isoforms: Wnt13A, Wnt13B and a new form Wnt13C (Struewing IT et al. 2006; Bunaciu RP et al. 2008). Wnt13C mRNA consists of exons 1 and 4-7 with the entire exon 2 skipped during an alternative splicing (see Figure 1.2A). Also, Wnt13A was shown transcribed from the P2 promoter, while mRNAs of Wnt13B and Wnt13C were generated using P1 promoter (Figure 1.2A, Struewing IT et al. 2006).

Two translational start codons were found in Wnt13B and C mRNAs: Met1 and Met74, so Wnt13B mRNA gave rise to a long form and a short form at protein levels, resulting in the detection of a protein doublet by western blotting, in contrast to Wnt13A with one single band of 41KD in size. This result was confirmed by site mutagenesis: mutant M1L-Wnt13B, where Met1 was replaced by leucine1, only generated the short form of Wnt13B (S-Wnt13B); however, mutant M74L-Wnt13B, where Met74 was replaced by Leucine74, only generated the long form (L-Wnt13B). Compared to Wnt13B mRNA, Wnt13C sequence had a deletion of 71 nucleotides corresponding to exon 2, leading to a change in the open reading frame of exon 4 and formation of a stop codon (Figure 1.2 B). Consequently, Wnt13C encoded the same protein as S-Wnt13B from the second translational start site (Met74), plus a very short peptide of 30 amino acids in length from the first start site (Met1), but only S-Wnt13B band was able to be seen by immunoblots (Struewing IT et al. 2006; Tang T et al. 2008).

The three Wnt13 isoforms displayed different subcellular localizations: Wnt13A, like most of other secreted Wnt members, was mainly retained in endoplasmic reticulum (ER), and underwent N-glycosylation during post-translational processing. In contrast, Wnt13B was not secreted but associated with intracellular fractions. Moreover, L-Wnt13B and S-Wnt13B were shown targeted to mitochondria and to the nucleus, respectively. Thus Wnt13 forms with S-Wnt13B alone, including Wnt13C and M1L-Wnt13B were localized in nucleus; however, Wnt13 forms with L-Wnt13B alone, like M74L-Wnt13B was localized in mitochondria due to N-terminus containing mitochondrial targeting sequences. Wnt13B, which had both long and short forms, was tightly associated with mitochondrial membranes and was able to induce the changes of mitochondrial morphology (from reticular to fragmented morphology) (Struewing IT et al. 2006).

1.2.3 Wnt13 expression and function

1.2.3.1 In humans

1.2.3.1.1 Human hematopoiesis

Wnt13 transcripts were detected by RT-PCR in thymus in human fetus and different hematopoeitic cell lines, including B-cell lines (Daudi, Raji, and Ramos), a T-cell line (Jurkat), myeloid cell lines (KG-1 and KG-1a), and erythroid cell lines (HEL and TF1), but not primitive stem/progenitor cells (CD34+Lin-), implying Wnt13 was likely expressed in committed progenitor cells and /or mature cells (Van Den Berg DJ et al. 1998). However, the Wnt13 primers used for PCR amplification can not distinguish the Wnt13 isoforms in this report, so the Wnt13 expression reflected the total levels of

Wnt13 isoforms. Also, Wnt13A-transduced CV-1 cells were used to test the biological activity: Wnt13 was shown to increase greatly the numbers of mixed colony-forming units (CFU-MIX), colony-forming units-granulocyte macrophage (CFU-GM), and burst forming units–erythoroid (BFU-E), indicating Wnt13 gene may be a new group of hematopoietic factors (Van Den Berg DJ et al. 1998).

Moreover, our laboratory further proved that all three Wnt13 isoforms were expressed in different human hematopoeitic cells including monoblastic U937 cells, erythroblastic K562 cells, and monocytic THP1 cells, with Wnt13C level being the highest compared to Wnt13A and B, by using specific primers for Wnt13A, Wnt13B and Wnt13C in realtime PCR reactions. In U937 cell line, Wnt13B and Wnt13C mRNAs were up-regulated while Wnt13A was down-regulated during cell differentiation towards monocyte/macrophages; however, in K562 cell line, the mRNA levels of all three Wnt13 forms were increased with cell differentiation to megakaryocytes. And the expression of Wnt13B and C, not Wnt13A were correlated to transcription factor MAF-B level in both U937 and K562 systems (Bunaciu RP et al. 2008). This finding demonstrated differential regulation of Wnt13 forms and Wnt13 isoform switch during U937 cell differentiation, further suggesting that Wnt13 forms may be new players in leukemic differentiation process towards monocyte/macrophages.

1.2.3.1.2 Human cancers

The Wnt13B mRNA was found in some cancer cell lines, such as Hela (cervical cancer), MKN28 and MKN74 (gastric cancer) (Katoh M et al. 1996). The Wnt13

expression was also detected by RT-PCR in human mammary epithelial cells, and breast cancer cell lines (BT-20, T-47-D, BT-474, MCF-7, MDA-MB-468, MDA-MB-453) as well (Benhaj K. et al. 2006). Besides, Wnt13 was shown to be expressed in 2 glioblastoma cell lines, 10 head and neck squamous cell carcinomas (HNSCC) cell lines, and 2 B cell tumor cell lines by using RT-PCR (Rhee C. et al. 2002). Wnt13 transcripts were detected in 4 cases out of 12 acute lymphoblastic leukaemia (ALL) cases using RT-PCR (Khan NI et al. 2007), and Wnt13 isoforms are also expressed in leukemic cell lines (Bunaciu RP et al.2008). In addition, Wnt13 transcripts were found in rat ovarian surface epithelium using in situ hybridization as well as in human ovarian cancer lines (SKOV-3, HEY, CAOV-3. OVCAR, SW626) using RT-PCR (Ricken A. et al. 2002). Although all these results imply the association between the levels of Wnt13 protein expression and human carcinogenesis, one limitation that most of these studies share is that the methodology they used could not distinguish the isoforms of Wnt13.

However, some other studies did specify the differential Wnt13 isoforms. The Wnt13 mRNA was shown up-regulated in 2 of 8 cases of primary gastric cancer by using matched tumor/normal expression array analysis where Wnt13 probe could detect both Wnt13A and B; in adult gastric epithelial cells, Wnt13A mRNA was the only form to be shown preferentially up-regulated in a case of primary gastric cancer by RT-PCR, and injection of synthetic mRNA of Wnt13A instead of Wnt13B was able to induce axis duplication in Xenopus embryos, suggesting that the Wnt13A may be positive regulator of Wnt/β-catenin pathway and its up-regulation may be related to tumor formation in gastric cancer (Katoh M et al. 2001). However, the activated effect on canonical signaling of Wnt13A is cell type specific, since Wnt13A failed to increase β-catenin/TCF activity

in HEK293 cells (Struewing IT et al. 2006). In addition, another report from Katoh has showed that Wnt13A mRNA, but not Wnt13B mRNA, was expressed in MCF-7 cells (breast cancer), NT2 cells (teratocarcinoma) and MKN45 cells (gastric cancer), further proved that differential expression of Wnt13 forms in carcinogenesis (Katoh M. 2001). Our laboratory also illustrated the differential expression and regulation of Wnt13 isoforms during leukemic cell differentiation: in U937 cell line, Wnt13B and Wnt13C mRNAs were up-regulated while Wnt13A was down-regulated during cell differentiation towards monocyte/macrophages (Bunaciu RP et al. 2008).

1.2.3.1.3 Human type II diabetes:

In 2008, Lee SH et al reported that using immune-histochemistry approach (Wnt13 antibody: AF3900, from R&D), Wnt13 and other canonical Wnt signaling members (such as TCF3) were absent from islets of nondiabetic individuals, but were greatly up-regulated in islets from type II diabetic patients, and Wnt13 was robustly expressed in both β-cells and α-cells of diabetics (Lee SH et al. 2008).

1.2.3.1.4 Inflammation in gastrointestinal tract

You J et al examined the expression of Wnt pathway-related genes in patients with ulcerative colitis (UC) by Wnt-specific microarray analysis, and they found that Wnt13 expression in colonic mucosa from patients with inflammatory bowel disease (IBD) was around six fold higher than normal colonic mucosa (You J et al. 2008), but this finding was not confirmed by RT-PCR using specific primers.

Moreover, Tanaka A et al took advantage of suppressive subtractive hybridization (a technique combining suppressive PCR and subtractive hybridization) to compare mRNA populations from biliary epithelial cells (BEC) isolated from patients with primary biliary cirrhosis (PBC) to BECs from normal samples, and they identified that one of 71 cDNA clones which have higher transcription levels in PBC patients, has 98% homology to human Wnt13 gene, but the paper did not specify Wnt13 isoform and the detail of the increase by Wnt13 (Tanaka A et al. 2001).

1.2.3.1.5 Others

Bisgaard AM et al described a case of deletion in chromosome 1(1p13.1 and 1p21.1) with breakpoints: the patient was a 13-year-old girl with severe mental retardation, short stature, dysmorphic appearance, and iris coloboma. The authors hypothesized that this phenotype in eyes might be contributed by Wnt13, as Wnt13 maps to 1p13.2 and Wnt13 is expressed in the optic vesicles during eye development (Bisgaard AM et al. 2006), but no evidence was given to support this hypothesis in the paper.

The Wnt13 mRNA was also detected by RT-PCR in human bone marrow stromal cells (hMSCs), also known as bone marrow-derived mesenchymal stem cells, which have the potential to differentiate into various types of cells such as osteoblasts and adipocytes (Shen L et al. 2009). Due to the lack of the information about Wnt13 primers in this paper, it is unknown whether the Wnt13 mRNA the authors detected represented the total Wnt13 or a specific isoform. In this study, there was no significant gender difference in Wnt13 expression; however, the Wnt13 mRNA demonstrated a trend of higher levels in young group, and for the hMSCs from women, the mRNA expression of Wnt13 was

inversely correlated with age, suggesting that Wnt13 gene expression may be affected by age (Shen L et al. 2009).

1.2.3.2 In animals

Wnt13 functions as the stem cell factor for neural or retinal progenitor cells during embryogenesis (Zakin LD et al. 1998; Nakagawa et al. 2003; Kubo F. et al. 2003). Unlike in humans, Wnt13 isoforms have not been specified in animals, so Wnt13 in animal studies are usually referred to ortholog of human Wnt13A.

1.2.3.2.1 Embryogenesis and organogenesis

During embryogenesis in mice, xenopus, chicken, and zebrafish, the *Wnt13* expression is similarly characterized by a dynamic pattern at different developing stages (Zakin LD et al. 1998; Jasoni C et al. 1999; Ng JK et al. 2002).

For mice, Wnt13A expression was detectable in the mouse embryonic mesoderm during gastrulation by using in situ hybridization. At later stages, robust expression of Wnt13A transcripts was found in neuroepithelium of the developing brain (as a narrow line of the dorsal midline of the diencephalon and mesencephalon), the heart primordia, the periphery of the lung bud and the otic and optic vesicles, suggesting Wnt13A may partially overlap with other Wnt genes in controlling mesoderm specification and embryonic patterning of brain, heart, or lung (Zakin LD et al. 1998).

1.2.3.2.2 Retinal development and degeneration

According to Jasoni C et al, chicken *Wnt13* was shown to be expressed in the developing eye with a two-phase pattern using in situ hybridization: the earlier expression was restricted within the pigment epithelium, and the later expression was confined to the lens and ciliary margin. Also strikingly, whole-mount hybridization experiment showed that *cWnt13* was restricted to the proliferating lens epithelium and absent from the quiescent, differentiating annular pad, and in vitro BrdU incorporation studies indicated the correlation between cell division and Wnt13 expression in the lens (Jasoni C et al. 1999), but the authors did not give specific information of the RNA probe for cWnt13 in this paper. Later, a study showed that dissociated retina cells formed the correctly laminated layer in Wnt13A-conditioned media, suggesting that Wnt13A may play a role in the formation of laminar structure in the retina (Nakagawa et al. 2003). Kubo F et al revealed that Wnt13A was visible in the marginal retina by in situ hybridization, and overexpressing Wnt13A by in ovo electroporation in central retina inhibited neuronal differentiation (Kubo F. et al. 2003). Also, the retina progenitor cells (RPC) in chicken ciliary marginal zone (CMZ) showed prolonged proliferation in Wnt13A-conditioned media in vitro, suggesting Wnt13A serves as a stem cell factor in the retina (Kubo F. et al. 2003). In contrast, in vivo studies by Cho SH et al showed another case: canonical Wnt signaling reduced RPC proliferation in vivo, and viral infection of Wnt13A interfered with the maintenance of retinal progenitor gene (RPG) expression, resulting in the conversion of retina cells into the peripheral fates of the ciliary body and iris (Cho SH et al. 2006). A recent report showed that Wnt13A induced the formation of both CMZ and iris/ciliary epithelium, which was mediated by Hairy1, a Notch signaling effector

(Kubo F et al. 2009). All these data demonstrate that Wnt13A plays a crucial role in controlling retina cell fate.

In addition to eye development, Wnt13 has been also shown to be involved in retina degeneration and regeneration. Wnt13 transcripts were detected during rod and cone photoreceptor death in mice by quantitative PCR (primer for Wnt13 was unable to distinguish Wnt13 isoforms), implying that Wnt13 might be a potential mediator in retina degeneration (Yi H. et al. 2007). Also, Wnt13 transcripts were found clearly expressed in the newt lens-regenerating iris tissue by RT-PCR, and local Wnt13 activation by lens removal or FGF-2 injection was restricted to the dorsal iris, indicating dorsal-specific activation of Wnt13 determines the dorsal iris-limited step during lens regeneration in the newt eye (Hayashi T et al. 2006).

1.2.3.2.3 Limb initiation

Kawakami Y. et al reported that Wnt13A-infected fibroblasts were able to induce Fgf-10 expression in lateral plate mesoderm, and to generate an ectopic limb when implanted in the flank of chicken embryo, which was mediated by stabilizing cytosolic and membrane β-catenin (Kawakami Y. et al. 2001). Similarly, in zebrafish embryos, Wnt13(Wnt2ba, which has 76% homology to human Wnt13A) loss-of-function experiments were performed by injection of Wnt13 morpholino oligonucleotides, and resulted in 75% embryos lacking pectoral fins, and this paper also showed Wnt13 was the upstream of Tbx5 and Fgf 10 to initiate limb outgrowth (Ng JK et al. 2002).

1.2.3.2.4 Liver specification

Ober EA et al found that *prometheus (prt)* gene mutation caused an absence or remarkable reduction in liver formation during the early stages of zebrafish development, which was a transient but dramatic phenotype. Indeed, *prt* gene encodes Wnt2bb, which is another Wnt13 ortholog in zebrafish (89% identical to Wnt2ba, and 77% homology to human Wnt13A and B), and knock-down of *wnt2bb* function by injecting morpholino antisense oligonucleotides resulted in a complete absence of liver, indicating a positive role for Wnt13 in zebrafish liver specification (Ober EA et al. 2006).

1.2.3.2.5 Kidney development

The *Wnt13* gene was also detected in the kidney of murine embryos using in situ hybridization (Lin Y et al. 2001). And isolated kidney mesenchyme which was placed on the monolayer of cells expressing secreted Wnt13A, failed to induce tubulogenesis, but ureteric bud cultured with Wnt13A-expressing cells showed promoted growth, branching and kidney reconstitution (Lin Y et al. 2001). Another report showed that canonical Wnt signaling was detected in branching ureteric bud tips and emerging S-shaped bodies in TCF-LacZ transgenic mouse kidney, and Wnt13A transfection increased Topflash activity greatly in MK4 cells (murine mesenchyme), which suggests that Wnt13 might be involved in the branching nephrogenesis in fetal kidney (Iglesias DM et al. 2007).

1.2.3.2.6 Vasculature

During organogenesis, zebrafish Wnt2bb was detected using in situ hybridization at the onset of somitogenesis in the posterior lateral plate mesoderm (LPM),

predominantly in cells fated to give rise to the endothelial and blood lineages, and expressed in the developing vasculature, endocardium, atrioventricular valve in the later stages (Ober EA et al. 2006).

1.2.3.2.7 Other organ development

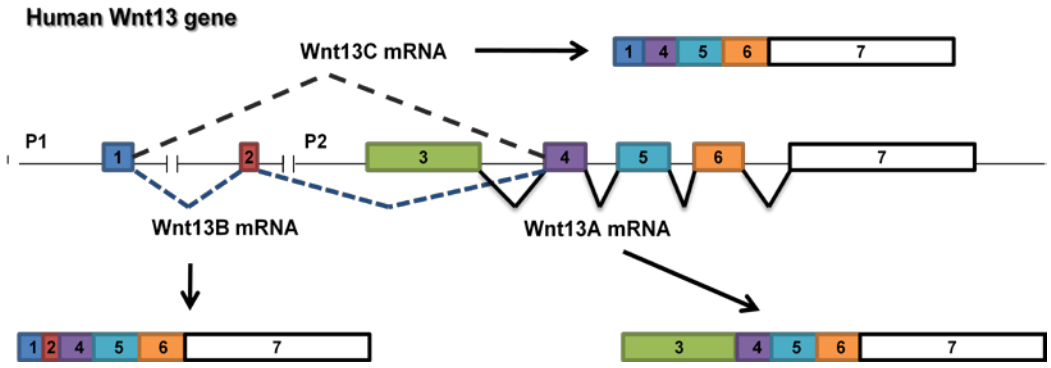
In xenopus, knockdown of Wnt13 by injecting different morpholino oligonucleotides resulted in a hypoplastic stomach as well as in hypoplasia of the pancreas, suggesting a positive role of Wnt13 in controlling proliferation of stomach and pancreas (Damianitsch K et al. 2009). In skeletal development, Wnt13 mRNA levels were decreased by 60% in human dermal fibroblasts exposed to demineralized bone powder (DBP) for 3 days, which is the first evidence that Wnt2b may influence post-natal chrondrocyte differentiation induced by DBP (Yates KE et al. 2004).

It was reported that the conventional Wnt13 knock-out mice did not show obvious phenotype (van Amerongen R et al. 2006), but this information was gained through their personal communication with Yamaguchi T without any description of how these knock-out mice were generated. Based on this result, either the role of Wnt13 might be not critical for development, or the deficiency of Wnt13 functions might be compensated by other Wnts.

Goss AM et al generated Wnt2 null mice, Wnt2b null mice, and Wnt2/Wnt2b double knockout mice to study the function of Wnt2 and Wnt13 in the development of the anterior foregut (Goss AM et al. 2009). In this study, Wnt2b null mice were viable without showing obvious phenotypes, which is consistent with the result discussed in van Amerongen R's review (van Amerongen R et al. 2006). Yet, most Wnt2 null mice were

cyanotic at birth and died perinatally; histological analysis in these mice showed remarkable lung hypoplasia, with poor development of the lung mesenchyme which resulted in dilated and abnormal vascular endothelial plexus by birth; these mice also displayed reduced cell growth in epithelial and mesenchyme cells and low levels of transcription factor important for lung development. Compared to Monkley SJ's finding showing placental defects with visible hematomas, abnormal large maternal blood pools, and reduced number of fetal capillaries in Wnt2 knockout mice (Monkley SJ et al. 1996), this new paper discovered the novel function of Wnt2 in lung development. Moreover, the Wnt2/Wnt2b double knockout mutants in this paper revealed complete failure of lung and tracheal development due to specific loss of lung progenitor specification in the foregut endoderm. The findings described above suggest the positive role of both Wnt2 and Wnt2b in specifying lung progenitors in the mouse foregut; yet, the difference between Wnt2 and Wnt2b is that the function of Wnt2 is not redundant whereas the role of Wnt2b may be, at least partially, compensated by Wnt2. In contrast, other organs derived from the foregut endoderm, including the thyroid, liver, pancreas and kidney, were unaffected by Wnt2/Wnt2b double knockout. This result seems to be contradictory to the findings by Ober EA et al: mutation in Wnt2bb gene caused a transient absence of liver formation in zebrafish development (Ober EA et al. 2006), which may be explained by the differential effects of Wnt2b in distinct species. Also, the phenotype of Wnt2bb knockout in zebrafish was transient, which implies that Wnt2bb deficiency may cause dramatic defects in liver specification at the early stage, but this deficiency might be compensated by other Wnts like Wnt2 in the later stage.

A)



B)

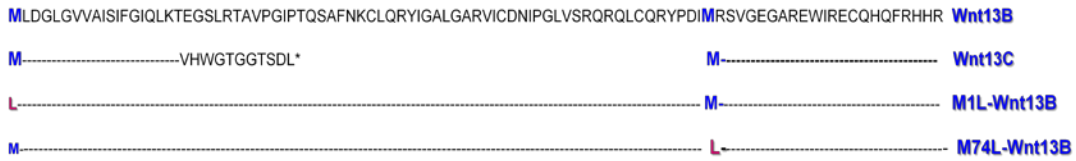


Figure 1.2 Three Wnt13 isoforms are generated via alternative promoter, alternative splicing, and alternative translational start codons. (A) The schematic human Wnt13 gene is shown, and Wnt13A, B and C mRNAs are produced through different promoters and alternative splicing sites. (B) N terminal sequences of Wnt13B, Wnt13C forms as well as M1L-Wnt13B, M74L-Wnt13B mutants, showing a long form and a short form at protein level can be generated via alternative translational start sites (modified from Struewing IT et al. 2006).

1.3 Cell apoptosis

1.3.1 Definition and features

Cell death is a programmed event during development and tissue turn over, or the ultimate consequence of the cell upon toxic or pathologic insults (Leist M et al. 1997). So far, at least three major types of cell death have been described: autophagy, a process characterized by portion of cytoplasm (including organelles) which is enwrapped in double-membrane vacuoles and targeted for lysosomal degradation; necrosis is a passive, pathological, catabolic process, which results from cell response to extreme challenges or toxic stimuli; and apoptosis, a form of programmed cell death, is an active process in need of energy, which can occur under both physiological and pathological circumstances (Renvoizé C et al. 1998; Galluzzi L et al. 2008). Besides, there are other forms of programmed cell death which are caspase-independent, such as Wallerian degeneration (nervous system), excitotoxicity (neurons), erythropoiesis, and cornification (epidermis) (Melino G et al. 2005; Kroemer G et al. 2009).

"Apoptosis" is a Greek word, originally meaning falling-off leaves from the tree in autumn. This term was anciently used by Hippocrates of Cos back in the 4^{th} century BC, and reused by Kerr JF et al to describe a morphologically distinct form of cell death (Diamantis A et al. 2008; Kerr JF et al. 1972).

The striking morphological changes make apoptosis easy to be identified *in vitro*. In nearly all cell types, apoptosis occurs in scattered, single cells or small cell clusters in a tissue which is actively regulating its own homeostasis. Once the cell is committed to suicide, chromatin condenses into dark crescentic masses adjacent to nuclear envelops,

leading to pyknosis, one of the most typical features in apoptosis. At the same time, the cell shrinks abruptly due to the loss of fluid, possibly because of blocked Na^+-K^+-Cl^- cotransporter system, and with cell shrinkage, the cytoplasm thereby becomes dense and the organelles are more tightly packed. Convolutions of plasma and nuclear membranes take place progressively with chromatin condensation, leading to a bubbling or extensive blebbing surface. Minor modification of cytoplasmic organelles is found, and the cell is segregated into apoptotic bodies which contain cytoplasm and cytoplasmic organelles with or without nuclear fragments, and the integrity of plasma membrane is remained. Subsequently, these apoptotic bodies are soon phagocytosed by professional phagocytes such as macrophages and dendritic cells, or by amateur phagocytes in the neighboring tissue such as epithelial cells and mesangial cells, and eventually degraded within lysosomes (Maderna P et al. 2003; Monks J et al. 2003). Interestingly, no inflammatory reaction is found during the process of apoptosis and phagocytosis of apoptotic bodies, since apoptotic cells keep plasma membrane intact so that no cellular components leak into surrounding tissue, and they are quickly engulfed by other cells which are not releasing inflammatory mediators (Allen RT et al. 1997; Elmore S. 2007)

In contrast to apoptosis, autophagy and necrosis have distinct morphological characteristics. Autophagy is featured by intracellular accumulation of autophagic vacuoles; however, necrosis is characterized by cellular swelling, lysed nucleus, rapid loss of cellular membrane integrity and diffuse inflammation (Allen RT et al. 1997; Loos B et al. 2009).

1.3.2 Apoptotic pathways and mechanisms

The mechanisms of apoptosis are complex and sophisticated, involving a lot of highly-regulated molecular events. So far two main apoptotic pathways, intrinsic pathway and extrinsic pathway, have been described in eukaryotic cells (Blank M et al. 2007; Elmore S. 2007), which are illustrated in Figure 1.3.1.

1.3.2.1 Intrinsic pathway

1.3.2.1.1 Overview

The intrinsic apoptotic signaling requires a variety of non-receptor stimuli that emanate intracellular signals converging at the mitochondria (Blank M et al. 2007). Mitochondria play a crucial role in cell apoptosis in mammals, because 1) the induction of mitochondrial outer membrane permeabilization (MOMP) is the pivotal event in the process of apoptosis; 2) the release of apoptogenic proteins, such as cytochrome c, from the mitochondrial intermembrane space to cytoplasm results in downstream execution phase apoptosis (Tsujimoto Y et al. 2000; Spierings D et al. 2005).

Two nonexclusive models may explain the induction of MOMP: 1) as shown in Figure 1.3.1, the pro-apoptotic Bax or Bak, assemble by homo-oligomerization and then create pore-like structures in the outer membrane (OM) without affecting the function of the inner membrane (IM) and the matrix. The Bax and Bak are members of Bcl-2 family, which is described in 1.3.2.3.1. 2) Some signals, like Ca^{2+} overload and increased ROS, induce the opening of permeability transition pore complex (PTPC) which consists of voltage-dependent anion channel (VDAC) in the OM and adenine nucleotide translocator (ANT) in the IM, and osmotic forces drive water into the matrix, resulting in swelling

and the ultimate rupture of both membranes (Spierings D et al. 2005; Garrido C et al. 2006).

Once MOMP occurs, the mitochondria undergo the loss of the membrane potential and release a variety of pro-apoptotic proteins to the cytosol. One of the apoptotic proteins is cytochrome c, which functions as an electron shuttle between complex III and complex IV of the respiratory chain. And in mitochondria, most of cytochrome c (85%) is trapped within the cristae and associated with cardiolipin while the rest is free in the intermembrane space (IMS) or loosely attached to IM (Garrido C et al. 2006; Schug ZT et al. 2009). As shown in Figure 1.3.1, upon MOMP, cytochrome c is released into the cytoplasm, where it binds and activates the apoptotic protease-activating factor (Apaf)-1, leading to the formation of a heptameric caspase-activating complex with deoxyadenosine triphosphate (dATP) and procaspase-9, termed the apoptosome. Each apoptosome recruits seven dimers of procaspase-9, and provides a platform that efficiently cleaves and activates caspase-9. In turn, activated caspase-9 induces the cleavage of executioner caspase-3, 6 and 7, which results in later-on execution phase of apoptosis (Wang Z et al. 2005; Jin Z et al. 2005).

Besides, the induction of MOMP also leads to the release of another type of apoptogenic factors including apoptotic inducing factor (AIF), endonuclease G (endoG) and Omi/HtrA2 to promote caspase-independent cell death (CICD) (Blank M et al. 2007). AIF, a mitochondrial flavoprotein, normally resides in IMS; however, upon apoptotic stimuli, AIF translocates from the mitochondria to the nucleus and induces peripheral chromatin condensation and large-scale DNA fragmentation (stage 1) (Donovan M et al. 2004). Likewise, endoG, a mitochondrial enzyme, also translocates from IMS to the

nucleus to cause oligonucleosomal DNA fragmentation (DNA laddering; stage 2) secondary to the first stage caused by AIF (Donovan M et al. 2004). Mammalian Omi/HtrA2, a serine protease with homology to the bacterial high temperature requirement A (HtrA) heat shock protease, can also induce CICD but the mechanism remains unclear (Donovan M et al. 2004).

1.3.2.1.2 Inducers of intrinsic pathways

The signals to induce the intrinsic apoptotic pathway include, but not limited to, oxidative stress, DNA damage, endoplasmic reticulum (ER) stress, cytoskeletal damage, hypoxia, loss of cell adhesion, toxins, macromolecular synthesis inhibition, viral infection, as well as the withdrawal of certain growth factors, hormones and cytokines (Blank M et al. 2007; Elmore S. 2007).

1.3.2.1.3 Regulators of intrinsic pathways

1.3.2.1.3.1 B cell leukemia/lymphoma (Bcl) -2 family

The *Bcl-2* gene was first discovered because of its involvement in the t(14;18) chromosomal translocations in a cell line derived from an acute lymphoblastic leukemia (Pegoraro L et al. 1984), and somatic mutations in translocated Bcl-2 was frequently found in lymphomas (Tanaka S et al. 1992) . Transgenic mouse studies showed that Bcl-2 protected thymocytes from cell apoptosis (Sentman CL et al. 1991), and Bcl-2 deficient

mice displayed massive apoptosis in thymus, spleen, and kidney (Veis DJ et al. 1993; Sorenson CM et al. 1995), indicating the critical role of Bcl-2 in suppressing apoptosis.

Later on, other proteins with structure similar to Bcl-2 were identified, and so far, there are more than 12 core members in the Bcl-2 family that range from inhibiting apoptosis to promoting apoptosis in their bioactivities (Youle RJ et al. 2008). The Bcl-2 family members share up to four Bcl-2 homology domains (BH1 to BH4). And they can be subdivided into three groups: the pro-apoptotic Bcl-2-associated X protein (Bax)/Bcl-2 homologous antagonist-killer protein (Bak) family members (Bax and Bak), the anti-apoptotic family members [Bcl-2, Bcl-X protein (Bcl-xl), Bcl-w, myeloid cell leukemia sequence-1 (Mcl-1) and Bcl-2 related protein A1A (A1A)], and the pro-apoptotic BH3-only proteins [Bcl-2 interacting mediator of cell death (Bim), Bcl-2 antagonist of cell death (Bad), BH3-interacting domain death agonist (Bid), p53 upregulated modulator of apoptosis (Puma), Noxa, death protein 5/Harakiri (Hrk), Bcl-2 interacting killer (Bik)] (Youle RJ et al. 2008). Bax and Bak have 3 core multi-BH domains: BH1, BH2, BH3, but anti- apoptotic members Bcl-2 have one more domain called BH4 that is required for Bcl-2 interacting with Bax; and BH3-only proteins do not contain any other BH domains but BH3 (Danial NN. 2007). The balance between pro-apoptotic members and anti-apoptotic members in Bcl-2 family governs the commitment of the cell to death, and constitutes a crucial switch during apoptosis.

BH3-only proteins function as initial sensors of specific apoptotic signals that are released from various cellular processes. In normal state, BH3-only proteins are in very low levels in the cytoplasm or in mitochondrial membranes; inactivated Bax is located in the cytoplasm or loosely attached to the mitochondria membrane; and Bak is located in

mitochondrial membrane, forming an inactivated complex with anti-apoptotic proteins like Bcl-2 (Lomonosova E et al. 2008). When BH3-only proteins are upregulated or activated by apoptotic stimuli, they liberate Bak or Bax either by binding and neutralizing Bcl-2 (Willis SN et al. 2007) or by binding and directly activating Bax (Gavathiotis E et al. 2008). Activated Bax, which then translocates from cytoplasm to mitochondria membrane, as well as activated Bak, which is originally located in mitochondria and released from Bak/Bcl-2 complex, undergo homo-oligomerization with multiple conformational changes to form pores at mitochondrial intramembrane (Lomonosova E et al. 2008). Once MOMP occurs, apoptogenic proteins such as cytochrome c are released from intermembrane space, activating caspase-9 through the formation of the apoptosome and thereby inducing the cleavage of executioner caspases, which results in the execution phase of apoptosis (Danial NN. 2007; Youle RJ et al. 2008). Therefore, Bcl-2 family consists of members with opposing functions in the apoptotic cascade, including "activators" such as Bax or Bak, "inhibitors or repressors" such as Bcl-2 or Bcl-xL, and "sensitizers or derepressors" such as BH3-only proteins (Bim or Bid) (Spierings D et al. 2005).

Bid, one of the BH3-only members, has a unique function in associating the intrinsic pathway with the extrinsic pathway. In the extrinsic pathway, Bid is cleaved by activated caspase-8, generating C-terminal truncated form t-Bid, which translocates to mitochondria and activates Bax or Bak to promote MOMP and downstream caspase activation (Youle RJ et al. 2008).

Bim is also one of the BH3-only members in the Bcl-2 family, and Bim exists in several isoforms: BimS, BimL, BimEL, BimAD and BimG. Bim is constitutively

expressed in various cell types but in an activated form through binding to the microtubule-associated dynein motor complex (Puthalakath H et al. 1999). Bim-deficient mice had abnormally high numbers of lymphoid and myeloid cells; B cells, T cells, monocytes and granulocytes were increased 2 to 4 fold in peripheral tissues from Bim-deficient mice than those from wildtype animals; and older Bim-deficient mice develop splenomegaly, lymphadenopathy, and autoimmune kidney disease, indicating that Bim is critical for apoptosis and homeostasis in the lymphoid and myeloid compartments (Bouillet P et al. 1999). Overexpression of Bim induced remarkably increased apoptosis in NIH 3T3 fibroblasts (Marani M et al. 2002) and in Hela cells (Herrant M er al. 2004). The mechanisms by which Bim induces apoptosis is not well understood; yet, Bim may function through interacting with and inhibiting Bcl-2 or Bcl-xL (Willis SN et al. 2007; Wang X et al. 2009) or through direct binding and activating Bax (Gavathiotis E et al. 2008). Antigens, cytotoxic agents, growth factor withdrawl and Reactive oxygen species (ROS) can activate Bim through various mechanisms: 1) by releasing it from the dynein motor complex or the anti-apoptotic Bcl2 proteins (Puthalakath H et al. 1999); 2) by increasing its mRNA level through transcriptional factors such as Forkhead Box O (FOXO) factors (Gilley J et al. 2003). Therefore, Bim serves as a key initiator of the intrinsic pathway of apoptosis, and the regulation of Bim expression and activity can tightly affect the downstream apoptotic events.

The intrinsic pathway is tightly controlled by the balance between pro-apoptotic proteins over anti-apoptotic proteins, especially the pro-/anti-apoptotic balance in the Bcl-2 family members. As described before, activated Bax can translocate to mitochondria from cytoplasm and oligomerize to generate pore-like structure in the OM,

while Bcl-2 or Bcl-xL can bind Bax or Bak after they insert into membranes and inhibit the oligmerization of Bax and Bak (Youle RJ et al. 2008). The pro- and anti-apoptotic Bcl-2 members also have opposite effect on MOMP: the pro-apoptotic Bax or Bak accelerate the openings of the VDAC channel, whereas the anti-apoptotic Bcl-xL closes VDAC (Shimizu S et al. 1999); moreover, the pro-apoptotic Bax can cooperate with ANT to yield an efficient composite channel, whereas the anti-apoptotic Bcl-2 inhibits ANT activity (Brenner C et al. 2000), proving that the Bcl-2 family members regulate the permeability of mitochondrial membrane, and thereby control both caspase-dependent cell death and caspase-independent cell death (Tsujimoto Y et al. 2000; Donovan M et al. 2004).

Besides cell apoptosis, Bcl-2 members have been also shown regulate other forms of programmed cell death, such as autophagy (Shimizu S et al. 2004). Both Bcl-2 and Bcl-xL bind and inhibit Beclin1, an essential autophagy protein through interaction of BH3 domains, while other BH3-only proteins can block the inhibition by Bcl-2 by competitively disrupting the interaction between Bcl-2 and Beclin1, and induce cell autophagy (Levine B et al. 2008). Therefore, Bcl-2 family members exert a duel role in regulating both apoptosis and autophagy.

1.3.2.1.3.2 Reactive oxygen species (ROS)

In addition to contain pro- and anti-apoptotic proteins, mitochondria are the major site producing ROS in aerobic cells, and thus another important regulator is the balance of oxidant/anti-oxidant defense (Hengartner MO et al. 2000; Ott M et al. 2007). To

protect against possible harmful consequences of ROS, cells also possess an endogenous anti-oxidative defense system consisting of reductants like glutathione (GSH), and enzyme systems that remove ROS through metabolic conversion (Ryter SW et al. 2007). For example, superoxide (O_2^-) can be converted by superoxide dismutases (SOD) to H_2O_2, which is in turn converted to H_2O and O_2 by catalase; in mitochondria, MnSOD, encoded by *SOD2* gene, is specifically used to catalyze the superoxide conversion (Kamata H et al. 1999). When the oxidative insults are not properly resolved by the cell anti-oxidative capacity, they can threaten cell homeostasis, and even cause intrinsic apoptotic cascade (Han H et al. 2004). ROS has been shown to induce mitochondrial permeability transition (Garrido C et al. 2006). Furthermore, it was found that the oxidation of cardiolipin, which is a mitochondria-specific anionic phospholipid, decreases the affinity of cytochrome c to cardiolipin and leads to the detachment of cytochrome c from the inner mitochondrial membrane, which may be another mechanism to explain the role of oxidative stress in cell apoptosis (Ott M et al. 2007). Also, peroxide contributes the induction of apoptosis by stimulating the activity of nuclear transcription factors to upregulate pro-apoptotic proteins or to inhibit anti-apoptotic proteins (Chandra J et al. 2000). In the other hand, some animal models of mitochondrial disease, such as the ANT1 knockout mice, showed remarkable increased production of hydrogen peroxide in mitochondria isolated from skeletal muscle, heart and brain (Esposito LA et al. 1999), suggesting that mitochondria dysfunction may lead to enhanced ROS production.

1.3.2.1.3.3 Others

As mentioned before, the morphology of the cell and its nucleus undergo dramatic changes during apoptosis. So does the mitochondria, and the striking morphological change in mitochondria may contribute to the induction of MOMP and the release of cytochrome c (Arnoult D et al. 2005). Mitochondria undergo extensive fragmentation upon apoptosis due to increased fission and reduced fusion. The pro-apoptotic Bak has been shown to be critical for mitochondrial fragmentation during apoptosis, which is inhibited by Bcl-2 (Brooks C et al. 2007).

Other factors have also been found to be involved in the regulation of the intrinsic apoptotic pathway. One type of negative regulators is inhibitors of apoptosis (IAPs), and the functional unit in IAPs is Baculoviral Inhibitor of apoptosis Repeat (BIR). X chromosome-linked inhibitor of apoptosis (XIAP) protein is believed to be the most potent IAPs, containing three BIR domains. XIAP directly binds and specifically inhibits the enzymatic activities of active caspase-3, 7 through the linker region between BIR1 and BIR2, whereas XIAP specifically inhibits active caspase-9 through BIR3 domain (Shi Y et al. 2002). The linker region and BIR3 may occupy the active site of caspases in a reverse orientation, thereby blocking the entry of substrates (Shi Y. 2002). XIAP also promotes proteasomal degradation of active caspase-3 via its ubiquitin-protein ligase activity (Blank M et al. 2007; Suzuki Y et al. 2001). The caspase-inhibitory activity of the IAPs, in turn, is blocked by IAP inhibitors, such as serine protease Omi/HtrA2 that can firstly sequester IAPs through its Reaper IAP-binding motif and subsequently degrade IAPs through its protease activity (Martins LM et al. 2002; Srinivasula SM et al. 2003), and second mitochondrial activator of caspases (Smac)/direct IAP binding protein

with low pH (Diablo) whose N-terminal residues (Ala-Val-Pro-Ile) can sequester the BIR domains of IAPs (Vaux DL et al. 2003). Upon the induction of MOMP, Omi/HtrA2 and Smac/Diablo are released from the inner member space of mitochondria to the cytoplasm, and compete with caspases for IAP binding (Blank M et al. 2007; Spierings D et al. 2005).

1.3.2.2 Extrinsic pathway

1.3.2.2.1 Overview

Unlike the intrinsic pathway, the extrinsic apoptotic signaling is dependent on transmembrane receptors, called death receptors, which binds to extracellular ligands. The death receptors are members of the tumor necrosis factor (TNF) receptor superfamily, featured by cysteine-rich extracellular domains and an 80 amino acid cytoplasmic tail, also termed “death domain”, which is critical for transducting death signal from the cell surface to inside the cells. The Fas Ligand/Fas and TNFα/TNFR are well-known ligand/death receptor pairs (Elmore S. 2007).

As shown in Figure 1.3, the interaction between the Fas ligand or TNF to Fas or TNFR leads to the recruitment of the adapter proteins Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD), which then binds procaspase-8 through dimerization of the death domain, generating a complex called the death-inducing signaling complex (DISC), in which procaspase-8 is processed, and subsequently caspase-8 is activated to directly trigger caspase-3- mediated execution phase of apoptosis (Wang Z et al. 2005).

The above process does not invovle Bcl-2 family members; however, in some systems such as spinal cord injury (Yu WR et al. 2009), kidney (Campbell MT et al. 2008) and various cancer cells (Bhattacharya K et al. 2009; Zhao Y et al. 2009), the extrinsic pathway can be linked to the intrinsic pathway through Bid, which is another group of pro-apoptotic Bcl-2 member. In the extrinsic pathway, the activated caspase-8 can cleave Bid with the generation of C-terminal truncated form t-Bid, which translocates to mitochondria and effectively activates Bax to promote downstream caspase activation through the induction of MOMP (Figure 1.3) (Youle RJ et al. 2008). Although MOMP is not required for the development of extrinsic apoptotic pathway, it exerts amplifying effect on apoptotic signals received from the death receptor at the cell surface (Blank M et al. 2007).

1.3.2.2.2 Inducers and regulators of the extrinsic pathway

The activation of extrinsic apoptotic pathway is initiated by the stimulation of death receptors by the binding of corresponding ligands. Besides Fas Ligand/Fas and TNFα/TNFR, others such as lymphotoxin-α/TNFR, Apo3L (TRAMP)/DR3, Apo2L (TRAIL)/death receptor (DR)4 and Apo2L (TRAIL)/DR5 have also been defined as ligand/death receptor pairs to trigger the extrinsic death signaling (Fulda S et al. 2004; Elmore S. 2007). Therefore, the expression of these death ligand/receptor pairs is important for the regulation of the extrinsic apoptotic pathway.

LPS is a highly pro-inflammatory molecule which can induce apoptosis through extrinsic apoptotic pathway in various systems, including endothelial cells (Bannerman

DD et al. 2003). Toll like receptor (TLR)-4 is the receptor of LPS, and LPS activates a caspase-8-initiated apoptosis dependent of FADD instead of TNFR1, Fas or DR3 in endothelial cells (Choi KB. 1998). In addition, the intrinsic apoptotic pathway is found to be required for LPS-induced apoptosis in human lung microvascular endothelial cells because the overexpression of Bcl-xL or deficiency of Bid can protect against cell death triggered by LPS (Wang HL et al. 2007).

Signal transduction of the extrinsic pathway can be negatively regulated by the cellular Fas-associated death domain like interleukin-1-converting enzyme inhibitory protein (c-FLIP), an enzymatically inactive homolog of caspase-8, which inactivates the extrinsic apoptotic pathway by competing with caspase-8 for interacting with FADD (Schultz DR et al. 2003; Wilson NS et al. 2009). So the expression of c-FLIP is negatively correlated with the activity of the extrinsic apoptotic pathway. Another protein called phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes-15 KD (PED/PEA-15) also block FasL, TRAIL or TNFα –induced apoptosis by interrupting FADD and caspase-8 interactions (Hao C et al. 2001). Additionally, the Bcl-2 family members are involved in the regulation of the extrinsic pathway, because 1) the pro-apoptotic tBid integrates the extrinsic apoptotic pathway with mitochondrial permeability; 2) in certain cell types, overexpression of the anti-apoptotic Bcl-2 and Bcl-xL by stable transfection blocks the apoptotic activity triggered by Fas ligand (Scaffidi C et al. 1998).

1.3.2.3 Effectors of apoptotic cascade (caspases)

Caspases are a family of highly conserved aspartate-specific cysteine proteases, and in the name, the “c” refers to cysteine used as the catalytic residues, and the “aspase” stands for cleaving their substrates behind aspartic acid residues. Yet, different caspases have different selectivity for cleavage through recognition of neighboring amino acid of aspartate (Alnemri ES et al. 1996). Each of these enzymes is synthesized as an inactive proenzyme in cells, which is proteolytically activated to form a heterodimetic catalytic domain (Alnemri ES et al. 1996). Caspases can either autoactivate themselves or are activated by other capases, followed by the initiation of caspase cascade and amplification of apoptotic signaling, which leads to rapid irreversible cell death (Elmore S. 2007).

To date, 15 mammalian members have been identified in the caspase family, and categorized into two subfamilies which are 1) inflammatory caspases (caspase-1, 4, 5, 11, 12, 13 and 14); and 2) apoptotic caspases that can be further grouped into initiator/apoptotic activator (caspase-2,8,9,and 10) and effector/apoptotic executioner (caspase-3,6,7) (Chowdhury I et al. 2008). Once receiving pro-apoptotic signals, the activation of the upstream initiator caspases (caspase-2, 8, 9, and 10) results in the proteolytic activation of the downstream executioner caspases (caspase-3, 6, and 7), and these effector caspases are the one that cleave the following protein substrates (Saikumar P et al. 1999; Saraste A et al.2000).

The substrates of caspases include: 1) cytoskeletal proteins such as actin and actin-binding proteins gelsolin and fodrin, which degradation by caspases leads to the

blebbing of the plasma membrane in apoptotic cells; 2) lamins and the nuclear matrix protein (NuMA), which function in maintaining nuclear integrity, and whose caspase-dependent cleavage is responsible for nuclear fragmentation; 3) the apoptotic chromatin condensation inducer in the nucleus (ACINUS), which is a modulator of chromatin condensation during apoptotic process; 4) the inhibitor of caspase-activated DNase (ICAD), when cleaved by caspases causing DNA fragmentation; 5) some DNA repair factors, such as poly(ADP-ribose) polymerase-1 (PARP-1) and the catalytic subunit of the DNA-dependent protein kinase (DNA-PK); 6) survival factors like MEKK-1, Akt-1, Bcl-2 (Blank M et al. 2006). Therefore, the activation of the effector caspases and their actions on protein cleavage initiate the apoptotic execution phase including DNA breakdown and morphological modifications.

Caspase-3 is thought to be the most important effector caspases. Caspase-3, activated by caspase-8, caspase-9, or caspase-10, preferentially activates caspase activated DNase (CAD) and ACINUS to induce DNA degradation and chromatin condensation respectively, and also cause cytoskeletal disassembly and the formation of apoptotic bodies (Elmore S. 2007).

Both the activity and the expression of caspases can be regulated. The natural inhibitors of caspase activity include IAPs, cytokine response modifier A (CrmA), p35 and v-FLIP, etc (Chang HY et al. 2000). Unlike IAPs which have mammalian homologs, virus proteins CrmA and p35 do not have cellular homologs so far, and they function as pan-caspase inhibitors through covalent modification of catalytic site of caspases (Shi Y. 2002). Posttranslational modifications also modulate the activity of caspases. For example, the phosphorylation of caspase-9 by Akt inhibits the activity of caspase-9; and

denitrosylation enhances the activity of mature caspase-3 (Earnshaw WE et al. 1999; Chang HY et al. 2000).

1.3.2.4 Other apoptotic features

1.3.2.4.1 DNA fragmentation

One of the earliest prominent features showing an irreversible commitment to cell death is DNA breakdown by Ca^{2+} - and Mg^{2+} -dependent endonucleases, ending up with DNA fragments of 180 to 200 base pair and multiple thereof, containing blunt end and single base 3' overhangs (Allen RT et al. 1997; Saraste A et al.2000). A number of caspase substrates are responsible for DNA breakdown during apoptosis, such as DNA fragmentation factor (DEF40)/CAD, which bind their inhibitory proteins DEF45/ ICAD respectively to form inactive heterodimers in normal condition, but these substrates can be selectively activated once cleaved by caspase-3 or other caspases, and then further breakdown double strand DNA to induce nuclear morphological changes (Saraste A et al.2000).

1.3.2.4.2 Phosphatidylserine translocation

To ensure apoptotic cell debris rapidly engulfed, there must be some changes in plasma membrane (PM) to create a recognition site by phagocytes (Allen RT et al. 1997). So another biochemical characteristic is the expression of cell surface markers; for example, phosphatidylserine, which is normally inward-facing in the cell lipid bilayer, translocates to outer leaflet of the PM during apoptosis, mediating phagocytic recognition as a ligand for phagocytes and subsequent elimination of apoptotic cells (Elmore S.

2007). In addition, other protein markers are also expressed in the cell surface when the cell undergoes apoptosis and phagocytosis, such as Annexin V, calreticulin, and thrombospondin-1 (Elmore S. 2007).

1.3.2.5 Apoptotic detection/quantification

One of commonly-used approaches to study the presence of apoptosis is to demonstrate the activation of caspases, either by western blot to show the caspase cleavage or by enzymatic assay to determine caspase activity (Saraste A et al.2000), and both are commercially available. The formation of DNA fragments has been applied for the detection of apoptosis: a characteristic “DNA ladder” pattern is evident in agarose gel stained with ethidium bromide and viewed under ultraviolet illumination; also, cell carrying DNA strand breaks can be visualized under microscope with the terminal transferase mediated DNA nick end labeling (TUNEL) assay (Saraste A et al.2000; Elmore S. 2007). Some markers such as Annexin V which are expressed at cell surface during apoptosis have also been used for detection of apoptotic cells commercially.

1.3.3 Regulation of apoptosis

The levels of apoptotic factors control the susceptibility of the cell to apoptosis. Based on the study from heterozygous and null mice of caspase-3, the expression of caspase-3 correlated with the sensitivity of T cells to apoptosis induced by etoposide (Sabbagh L et al. 2004). Human U937 cells treated by γ-interferon which can induce

caspase expression showed enhanced susceptibility to apoptosis triggered by gamma-irradiation or antitumor agents (Tamura T et al. 1996). The deregulation of apoptotic factors is highly related to tumorigenesis; for example, lymphomas were often found to bear chromosomal translocation of the anti-apoptotic gene *Bcl-2* resulting in hypomethylation and high-level expression of Bcl-2 gene (Hanada M et al. 1993); and the expression of the pro-apoptotic Bim at both mRNA levels and protein levels were shown significantly lower in chronic myeloid leukemia (CML) cells than that in normal cells (Aichberger KJ et al. 2005). The expression of apoptotic factors is also important for development (such as caspases) and function of immune system (such as FasL/Fas) (Kuida K et al. 1996; Ettinger R et al. 1995). The apoptotic cascade is controlled by the balance between pro-apoptotic proteins and anti-apoptotic proteins, and this regulation can occur in a variety of levels including transcriptional levels, post-transcriptional levels and translational levels.

1.3.3.1 Transcription levels

The gene expression can be controlled by transcription factors interacting with their specific DNA sequences with other co-activators/co-repressors (Lemon B et al. 2000). The expression of apoptotic factors such as caspases, Bcl-2 family members, and factors in the extrinsic pathway have been shown to be tightly regulated by certain transcription factors and cofactors.

1.3.3.1.1 Forkhead box O (FOXO) factors

FOXO proteins are a family of transcription factors with important roles in metabolism, cell cycle, stress tolerance and possibly lifespan (van der Horst A et al. 2007). FOXO family consists of FOXO1, 3a, 4 and 6, and FOXO proteins undergo post-translational modification like phophorylation, ubiquitination, acetylation and methylation which activates or inhibits FOXO activity (Huang H et al. 2007; Yamagata K et al. 2008). In the presence of growth factors, FOXO phosphorylation by Akt, serum and glucocorticoid inducible protein kinase (SGK), I-kappa B kinase (IKK) and cyclin-dependent kinase (CDK2) is inhibitory. However, under stress, the phosphorylation by JNK or MST1 is stimulatory, and can induce cell death in neurons (Barthélémy C et al. 2004; Lehtinen MK et al. 2006). When FOXO proteins are inactivated, they are excluded out of the nucleus and then degraded by proteasome; however, activated FOXOs stay in nucleus and initiate the transcription of their target genes, including DNA repair-related *Gadd45*, oxidative detoxification-related *SOD2* and *catalase*, cell cycle-related $p27^{KIP1}$, *cyclin D1* and *cyclin D2*, as well as apoptosis-related caspase-3, *FasL*, *Bim*, and *TRAIL* (Huang H et al. 2007).

FOXO1-deficient mice died at around embryonic day 11 due to branchial arch defects and abnormal vascular remodeling in the yolk sacs, indicating that FOXO1 is required for mouse vascular development (Furuyama T et al. 2004). Also, FOXO1, 3, 4 triple-knockout mice developed thymic lymphomas and hemangiomas (endothelial cell tumor), suggesting that FOXOs are tumor suppressors and specifically important for endothelial cell homeostasis (Paik JH et al. 2007). The tumor suppressing effect of FOXOs may be related to their pro-apoptotic functions. Also, constitutively activated

form FOXO1-TM has been shown to induce apoptosis in leukemia-derived cell lines via *TRAIL* (Kikuchi S et al. 2007); knock-down of FOXO3a by small interfering RNA abolished free fatty acid-induced hepatocyte apoptosis and Bim induction (Barreyro FJ et al. 2007), further indicating the promoting role of FOXOs in cell apoptosis.

FOXOs are also found to converge with the Wnt/β-catenin signaling; in 2005, Essers MA discovered a functional interaction between FOXO and β-catenin under oxidative stress, so a new model of Wnt/FOXO was then proposed: upon Wnt signaling which turns TCF on, or upon insulin signaling that turns FOXOs off, β-catenin favors interacting with TCF instead of FOXO to promote cell proliferation; in contrast, under oxidative stress conditions where FOXO activity is on, β-catenin preferentially binds to FOXOs to induce apoptosis or quiescence (Essers MA et al. 2005; Bowerman B. 2005).

1.3.3.1.2 p53

The transcription factor p53 is a tumor suppressor which is activated in response to DNA damage or oncogenic transformation, and its functions in inducing cell cycle arrest, cell death and DNA repair are well known (Harms KL et al. 2005). P53 can promote cell apoptosis through the following mechanism: 1) p53 transactivates target genes such as the pro-apoptotic Puma and Noxa to activate Bax both by directly interacting with Bax and by inhibiting anti-apoptotic Bcl-xL (Nakano K et al. 2001; Zhang Y et al. 2009); 2) p53 transactivates Bax, Bak and Bid as well as Apaf-1 to induce MOMP which is following by caspase activation (Miyashita T et al. 1995; Kannan K et al. 2001; Moroni MC et al. 2001; Schuler M et al. 2005). More recently, p53 has been demonstrated to induce apoptosis through a novel transcription-independent activity.

Upon stress, p53 translocates to mitochondria and functions as a direct apoptogenic factor to induce MOMP by neutralizing the inhibitory effects of Bcl-2 and Bcl-xL and by activating Bax/Bak (Mihara M et al. 2003; Chipuk JE et al. 2004). Also, p53 integrates its transcription activity with its transcription-independent activity to induce cell apoptosis by Puma, which is transcriptionally upregulated by nuclear p53, and also liberates p53 from Bcl-xL, allowing p53 to induce MOMP and cell apoptosis (Chipuk JE et al. 2005; Vaseva AV et al. 2009).

1.3.3.2 mRNA stability

The fate of mRNAs has been believed to be one of the key steps in regulation of gene expression, and the stability of mRNAs is closely controlled post-transcriptionally (Donnini M et al. 2004). The Bcl-2 mRNA harbors an evolutionary conserved A+U-rich element (ARE) containing several AUUUA motifs in 3'-untranslated region (UTR). The Bcl-2 ARE has a moderate destabilizing activity, which is greatly enhanced by apoptotic stimuli while greatly reduced by PKC stimulation, thereby decreasing or prolonging the half-life of Bcl-2 mRNA (Schiavone N et al. 2000; Donnini M et al. 2001). Besides ARE, the CA repeats located upstream of ARE also contribute to the degradation of Bcl-2 mRNA in the steady states (Lee JH et al. 2004).

In addition, some other members of Bcl-2 family also can be regulated at their 3'-UTR area. Similar to the Bcl-2 mRNA, the expression of the anti-apoptotic Bcl-xL is increased during apoptosis induced by UVA due to the enhanced mRNA stability through promoting the binding of 3'-UTR with RNA-binding proteins (Bachelor MA et al. 2004).

Another example is anti-apoptotic Bcl-w, which expression is down-regulated by miR-122, a hepato-specific microRNA, by being directly targeted at 3'-UTR (Lin CJ et al. 2008).

1.3.3.1 Translational regulation

Eukaryotic translation is composed of three phases: initiation, elongation and termination, and initiation, the rate-limiting step, is believed to be the most important and best studied process for translational regulation (Holcik M et al. 2005). Under physiological conditions, the majority of mature eukaryotic mRNAs undergoes translational initiation by a cap-dependent ribosomal scanning mechanism, which involves numerous eukaryotic initiation factors: eIF4F (cap-binding protein complex, composed of eIF4E, 4A and 4G), eIF2 (catalyzing transfer of methionyl-tRNA to 43S pre-initiation complex), and adaptor eIF3, etc (Graber TE et al.2007).

In contrast, in the context of apoptosis induced by cell stress, the cap-dependent translational initiation is impaired via distinct mechanisms including eIF2 phosphorylation, eIF4E sequestration, and eIF4G proteolysis by caspases. But the paradox is that *de novo* protein synthesis is needed during cell stress because the cell must decide to live or to die by producing either more anti-apoptotic proteins to recover itself or more pro-apoptotic proteins to sustain the death signal, so in this case, there is a switch between the cap-dependent translation to alternative mechanisms, such as ribosomal shunting, re-initiation and internal ribosome entry (Graber TE et al.2007; Kozak M. 2003).

The process of internal ribosome entry is a cap-independent translational mechanism. Instead of employing scanning of the preinitiation complex from the 5'-cap toward the start codon, internal ribosome entry requires the direct recruitment of ribosomes to a complex RNA structural element called the internal ribosome entry segment (IRES) located at 5'-untranslated region (UTR) of the mRNA, which is facilitated by IRES trans-acting factors (ITAFs) (Spriggs KA et al.2005; Marash L et al. 2005). For example, the mRNA of X-linked inhibitor of apoptosis (XIAP), which inhibits caspase-3 and 7 directly, has been demonstrated to contain the functional IRES motif, and the translation directed by XIAP-IRES is resistant to cell stress induced by γ-irradiation or serum starvation in Hela and H661 cell lines (Holcik M et al. 1999). So the transcription of XIAP is maintained in these tumor cells even under stress, which may be one of mechanisms that tumor cells employ to escape apoptosis. Another anti-apoptotic factor carrying IRES at 5'-UTR is Bcl-2, and Bcl-2 IRES activity is induced in response to stress, resulting in increased protein levels (Sherrill KW et al. 2004). However, besides anti-apoptotic proteins, numerous pro-apoptotic proteins initiate their translation through IRES mechanism upon apoptosis. C-myc is a proto-oncogene, but its ectopic overexpression can sensitize cells to apoptosis, and c-myc-deficient cells fail to undergo apoptosis induced by DNA damage (Adachi S et al. 2000). Therefore, c-myc is necessary for cell apoptosis. During apoptosis in Hela cells triggered by the extrinsic pathway initiator TRAIL or the intrinsic pathway inducer staurosporine, the general protein translation is inhibited while c-myc protein expression remains constrained, and c-myc IRES is active while there is no increase for c-myc mRNA levels and no change in c-myc half life, indicating c-myc protein synthesis is initiated via IRES mechanism during

apoptosis (Stoneley M et al. 2000). Also, IRES-mediated translation of Apaf-1, but not XIAP is enhanced during UVC irradiation induced cell death in HEK-293T cells (Ungureanu NH et al. 2006). Additionally, under etoposide-induced DNA damage, p53 synthesis is dramatically induced in MCF-7 cells, and this translational regulation can be at least partially explained by the identification of an active IRES site from p53 transcript (Yang DQ et al. 2006).

The death associated protein (DAP) 5, also named p97 and NAT1, a member of the eIF4G family lacking the eIF4E binding site, acts as a dominant negative form to prevent cell from apoptosis (Marash L et al. 2005). Yet, DAP5/p97 can be activated during apoptosis by caspase cleavage, yielding a C-terminal truncated protein of 86KD, which promotes IRES mediated translation of death proteins, including c-Myc, Apaf-1, DAP5 and XIAP (Henis-Korenblit S et al. 2000; Henis-Korenblit S et al. 2002). Therefore, the activation of DAP5 IRES under cell apoptosis creates a positive feedback loop to potentiate the translation of DAP5 itself and other death proteins when the general translation machinery is abrogated, in order to help the cell to determine to live or die under stress circumstances.

1.3.4 Biological significance

Cell death programs are believed to be key players in embryonic development, tissue homeostasis, and cellular responses to stress, and the role of apoptosis in physiological conditions is as important as its counterpart, cell mitosis (Blank M et al. 2007; Elmore S. 2007).

1.3.4.1 Development

Apoptosis is a common and conserved characteristic in the development of many mammalian organs or tissues, and apoptosis plays a critical role in the process of normal development in vertebrate, including neural tube closure, eye development, palate development, and deletion of Mullerian duct in males or Wolffian duct in females (Mirkes PE. 2002). As for neural development, the nervous system initially overproduces cells, which is then followed by the elimination of at least half of the original population through cell apoptosis, resulting in removal of unconnected neural cells, as well as optimization of synaptic connection and neurons (Nijhawan D et al. 2000). Furthermore, caspase-3 knockout mice showed profound defects in brain development featuring multiple indentations and ectopic cell masses, and these supernumerary cells were found primarily due to a decrease of cell apoptosis in the brain, which supports that caspase-3 mediated apoptosis is crucial during morphogenetic cell death in the brain development (Kuida K et al. 1996).

1.3.4.2 Immune system

Apoptosis is a central regulator of immune system for the maintenance of self-tolerance and tight control of lymphocyte populations, which includes negative selection of developing B cells (removing B cells expressing autoreactive B cell receptors), as well as positive and negative selection of developing T cells (Maniati E et al. 2008). Moreover, regulation of peripheral T cells is also dependent on cell apoptosis: T cell are in resting status in peripheral lymphoid organs until they receive stimulating signals

(antigen presentation); when immune response is over, activated T cell must be eliminated via apoptosis to keep the homeostasis of immune systems (Krammer PH et al. 2007). Additionally, cytotoxic T lymphocytes (CTL) can induce apoptosis in target cells through granzyme/perforin or Fas/Fas ligand, which is of vital importance to protect the host from pathogenic viruses (Osborne BA. 1996; Keckler MS et al. 2007). Deregulated immune cell death is able to give rise to severe consequences: an aberrant expansion of apoptotic cells leads to immunodeficiency like AIDS, whereas failed apoptosis of lymphocytes leads to lymphoma or autoimmune disorders such as autoimmune lymphoproliferative syndrome and autoimmune diabetes (Saikumar P et al. 1999; Maniati E et al. 2008).

1.3.4.3 Wound healing

Wound healing need a series of quick changes of specific cell populations functioning in preparing, depositing and maturing the wound. Different cell types are needed in specific stages, and once finishing their own missions, these cells have to be removed through apoptosis before the next step starts, which minimizes the inflammatory reaction and tissue injury. In the early stage of wound healing, apoptosis starts from 12 hours after wounding to eliminate inflammatory cells (neutrophils and macrophages); in the later phase, fibroblasts and endothelial cells are found to undergo apoptosis in a similar pattern, along with decreased cellularity and vascularity in wound during maturation phase. Also, deregulation of cell apoptosis in wound healing causes pathological damage to the tissue like hypertrophic scarring (Greenhalgh DG. 1998).

1.3.4.4 Carcinogenesis

A major cause of malignant transformation and tumor formation is the genetic abnormalities in pathways that regulate cellular growth and death. Indeed, suppression or failure of apoptosis is one of key features in cancer cells, and one of the central players in the development and progression of some cancers (Elmore S. 2007). Inhibited apoptosis not only is associated with the transformation process in tumor development, but also lead to the resistance of cancer cells to treatments such as chemotherapy. Thus FasL delivery by tissue-specific virus is based on tumor-specific translation of tumor-killing genes, and showed antitumor effect in prostate cancer cells and in mice (Li X et al. 2007).

Mutations of apoptotic factors affect either their activity or their expression. Apoptosis-related genes that draw most attentions in cancer research are *p53* and *Bcl-2*. Mutations of p53 have been found in nearly all the tumor types, and may be associated with half of all cancers, which makes p53 one of most prominent tumor suppressor (Amundson SA et al. 1998). The majority of p53 mutations is missense and located in the DNA binding domain, leading to disrupted p53 activity in cancers (Rose SL et al. 2003). The proto-oncogene bcl-2 was identified at the chromosomal break point of t(14;18) in patients with B cell follicular lymphomas, resulting in abnormally high-level expression of Bcl-2 at transcription levels (Sentman CL et al. 1991; Malagurneral L. 2004). Bcl-2 transgenic mice showed elongated survival of B cells and T cells, and more resistance to cell apoptosis induced by glucocorticoid, radiation and anti-CD3 (Sentman CL et al. 1991). Besides, other factors in apoptotic pathways are also related to tumorigenesis. Bax-deficient mice displayed accelerated tumor growth and abolished apoptosis (Yin C et al. 1997), and a human study also reported that 21 out of 41 case of primary colon

adenocarcinomas carried frameshift mutations within *Bax* gene, further proving that the relationship between genetic alternation of apoptotic molecules and carcinogenesis (Rampino N et al. 1997).

1.3.5 Endothelial cell apoptosis

1.3.5.1 Physiological importance

Vascular endothelial cells (ECs) form the inner lining of blood vessels and the heart, and regulate the permeability of blood vessels to leukocytes and inflammatory factors (Choy JC et al. 2001). During both physiological development and pathological conditions, neovascularization and the vessel regression are determined by the balance between proliferation and apoptosis of ECs; and in mature vessels, EC turnover is also tightly controlled to maintain vessel homeostasis (Mallat Z et al. 2000).

EC apoptosis have been shown to be required for vessel regression during normal vessel development and remodeling. Macrophage ablation transgenic mice showed eye defects due to persistence of normally transient remnants of hyaloid vessel system of the eye and persistence of the excessive papillary membrane, demonstrating that macrophage actively elicits endothelial cell apoptosis, leading to normal capillary regression, which ensures the proper development of the eye (Lang RA et al. 1993). The dead cells are thereby mobilized into the capillary lumen causing restriction and a block to blood flow. At this point, vascular ECs undergo a secondary apoptosis with a synchronous pattern, leading to another capillary regression to clear off all remaining cells in the affected segment (Meeson A et al.1996). Another example is that during the regression of the

corpus luteum in the guinea pig, there is rapid endothelial cell death in ovarian blood vessel with prominent apoptotic features (nuclear condensation and fragmentation) (Azmi et al. 1984), also indicating the relationship between endothelial cell apoptosis and vessel regression.

1.3.5.2 Pathological importance

As the vascular endothelium is critical for various normal conditions, endothelial cell apoptosis may be involved in diverse pathological circumstances like atherosclerosis and angiogenesis (Mallat Z et al. 2000; Dimmeler S et al. 2000).

Atherosclerosis is a multifactorial disease which develops in the arterial wall in response to numerous pathological insults and results in excessive inflammatory injury and fibro-proliferative plaque (Sima AV et al.2009). A number of evidences have shown increased EC apoptosis in atherosclerotic plaques compared with normal tissues (Choy JC et al. 2001). EC apoptosis may be induced by oxLDL, cytotoxic T lymphocytes (CTL), cytokines, ROS or local inflammatory mediators (Sima AV et al.2009). In the early stage of atherosclerosis, EC apoptosis induces loss of EC number and EC integrity, leading to enhanced vascular permeability, vascular smooth muscle cell migration and increased blood coagulation (Choy JC et al. 2001).

Angiogenesis is the process of neovasuclarization during both development and postnatal period, mediated by proliferation, migration and remodeling of differentiated endothelial cells. Unlike being mainly deleterious in atherosclerosis, EC apoptosis, counteracting proliferation, has an inhibitory function in tumor angiogenesis (Dimmeler S et al. 2000). Vascular endothelial growth factor (VEGF), which is a required growth

factor for angiogenesis, can efficiently block endothelial cell apoptosis through various mechanisms including: 1) inducing Bcl-2 expression (Pidgeon GP et al. 2001); 2) activating extracellular signal regulated kinase (ERK) 1 and ERK2 signaling and inhibiting stress-activated protein kinase (SAPK) /JNK signaling (Gupta K et al. 1999); 3) activating PI3-kinase/Akt pathway through VEGF receptor, fetal liver kinase-1(Flk-1)/kinase-insert domain-containing receptor (KDR) (Gerber HP et al. 1998). Moreover, administration of anti-VEGF monoclonal antibodies results in regression of tumor-associated vasculature in xenograft mouse models (Yuan F et al. 1996). Thus, VEGF becomes a privileged target for the control of angiogenesis in anti-tumoral goal, and anti-VEGF treatment has been applied in tumor therapy. A single infusion of the anti-VEGF antibody bevacizumab reduced blood perfusion of tumor, vascular volume, microvasucular density (MVD) and interstitial fluid pressure (IFP), as well as amount of circulating endothelial cells and progenitors in 6 patients with rectal cancer, which directly indicates the antivascular role of anti-VEGF therapy in human tumors (Willett CG et al. 2004).

1.3.6 Wnts/wnt signaling and cell apoptosis

1.3.6.1 Wnt proteins and cell apoptosis

Wnt proteins, serving as stem cell factors in development and adult homeostasis, are usually thought to have proliferating or anti-apoptotic functions. Nonetheless, the role of Wnts in cell apoptosis is more complex: generally speaking, in transformed cells, Wnts

show anti-apoptotic actions; in contrast, in normal cells, Wnts seem to have pro-apoptotic functions.

Transformed HEK293 cells stably transfected with Wnt3a and 5a showed reduced apoptosis induced by serum-starvation compared to cells transfected with vectors (Jia L et al. 2008). And Wnt1, 3a and 5a were found to prevent cell apoptosis in the uncommitted bipotential C2C12 cells, the pre-osteoblastic cell line MC3T3-E1, and bone marrow-derived OB-6 osteoblasts (Almeida M et al. 2005). However, Wnt7b was shown to mediate macrophage-induced programmed cell death in the developing mouse eye (Lobov IB et al. 2005). And the overexpression of Wnt5a led to increased apoptosis in thymocytes *in vitro* (Liang H et al. 2007). Wnt3a showed pro-apoptotic functions in the H9C2 cardiomyoblast line subjected to hypoxia reoxygenation (HR) injury (Zhang Z et al. 2009).

Our laboratory showed that nuclear Wnt13 forms significantly increased the appearance of apoptotic nuclei in BAEC. At the basal level, the caspase-3 cleavage was undetectable in BAEC transfected with different Wnt13 isoforms; however, the activated caspase-3 was observed when BAEC was treated with TNF-α (5ng/ml) for 12 hours, and under this condition, nuclear Wnt13 forms further increased the levels of caspase-3 activation, suggesting that nuclear Wnt13 forms enhance the susceptibility of endothelial cells to stimuli during apoptosis (Struewing IT et al. 2006).

All these studies show that the differential effects of Wnts on cell apoptosis are dependent on cell type and possibly cell status. And since Wnt13 have 3 different isoforms with distinct subcellular localizations which can underlie possible differential

effects of three Wnt13 forms, it is of our interest to further analyze the role of Wnt13 isoforms in apoptosis and to determine the mechanisms.

1.3.6.2 GSK-3β and cell apoptosis

GSK-3β, a multi-functional serine/threonine kinase, is the key regulator in Wnt signaling and insulin signaling, and also implicated in the development of a variety of human diseases including cardiovascular diseases, neurodegenerative disease, bipolar disorder and cancer (Luo J. 2009). Overexpression of GSK-3β was sufficient to trigger apoptosis in different types of cells, such as neurons, vascular smooth muscle cells, human umbilical vein endothelial cells (HUVEC), and astrocytes (Li M et al. 2000; Hall JL et al. 2001; Kim HS et al. 2002; Sanchez JF et al. 2003). And GSK-3β has been found to promote the intrinsic apoptotic pathway triggered by different cellular insults, such as DNA damage, ER stress, hypoxia, removal of NGF or BDNF, hypertonic stress, oxidative stress, mitochondrial toxins and ceramide, by regulating transcription factors like p53 that controls pro- and anti-apoptotic proteins, by promoting microtubule disruption, and by inducing mitochondrial disruption though activating pro-apoptotic members like Bax or degrading anti-apoptotic members like Mcl-1 (Beurer E et al. 2006; Forde JE et al. 2007). Moreover, the subcellular localization of GSK-3β was shown important for its functions. In embryonic stem cells (ESCs), GSK-3β shuttled between the cytoplasm and the nucleus through Akt signaling: Akt activity inactivated and exported GSK-3β to the cytosol; however, the decreased Akt signaling promoted accumulation of the active GSK-3β which inhibit c-myc through phosphorylation, resulting in differentiation instead of self-renewal in ESCs (Bechard M et al. 2009). Also,

in untreated human neuroblastoma cells, GSK-3β was mainly located in cytoplasm; however, the exposure of cells to different pro-apoptotic stimuli like serum-starvation, heat shock or staurosporine induced a rapid increase of nuclear GSK-3β (Bijur GN et al. 2001), further suggesting that the subcellular distribution of GSK-3β is dynamically regulated by pro-apoptotic or anti-apoptotic signals. On the other hand, Lithium, the inhibitor of GSK-3β, reduced mouse acute renal failure induced by LPS via attenuating inflammation and renal cell apoptosis (Wang Y et al. 2009), further indicating that GSK-3β has promoting role in cell apoptosis and inflammation.

Ironically, GSK-3β was also been found to prevent apoptosis in some conditions. For example, lithium was shown to sensitize primary rat hepatocytes to TNFα-induced apoptosis (Schwabe RF et al. 2002); also, inhibition of GSK3 by chemical or RNA interference led to glioma cell death, accompanied with c-myc activation, the upregulation of pro-apoptotic factors and destroyed NF-κB activity (Kotliarova S et al. 2008). The homozygous GSK-3β knockout mouse embryos were non-viable, and histological examination of GSK-3β-/- embryos showed multifocal hemorrhage in liver with severe apoptosis, and embryonic fibroblasts from GSK-3β-/- embryos had increased sensitivity to TNF-induced apoptosis, also indicating that GSK-3β is required for cell survival (Hoeflich KP et al. 2000).All the above observations make it difficult to clearly define the function of GSK-3β in cell apoptosis due to its range of conflicting roles, but in general, more evidence supports the pro-apoptotic function of active GSK-3β.

1.3.6.3 C-myc and cell apoptosis

The transcription factor c-myc is a proto-oncogene which constitutive expression results in the development and progression of tumors. c-myc transactivates cell cycle promoting genes like cdc25A, cdk4 as well as cyclin D2,-E, -A while suppresses cell cycle inhibiting genes such as *gas1*, *p15*, $p21^{cip}$ and $p27^{kip}$ (Gartel AL et al. 2003). Interestingly, increasing evidence shows that inappropriate c-myc expression not only promotes cell proliferation and oncogenesis, but it also can induce or sensitize cells to apoptosis (Hoffman B et al. 1998). For example, in IL-3 dependent myeloid cell line, enforced c-myc expression accelerated cell apoptosis in the absence of IL-3 (Askewe DS et al. 1991). Besides, other cell types like hepatocytes and epithelial cells can also be subjected to similar apoptotic responses induced by c-myc (Hoffman B et al. 1998). The underlying mechanisms implicated in c-myc-induced apoptosis are very complex: 1) c-myc stabilizes p53 by p19ARF; 2) c-myc upregulates the expression of pro-apoptotic Bax and Bim, while blocks the expression of anti-apoptotic Bcl-2 and Bcl-xL, resulting in cytochrome c release; 3) c-myc may cause DNA double strand breaks to induce apoptosis (Adhikary S et al. 2005; Hoffman B et al. 2008).

1.3.6.4 Other Wnt signaling components and cell apoptosis

Overexpression of Wnt receptors like Fz1 and Fz2 in COS7 and HEK293 cells resulted in increased cell apoptosis detected by TUNEL staining and DNA ladder assay (van Gijn ME et al. 2001). Also, *Xenopus* embryos injected with Xfz8 showed increased cell apoptosis with the involvement of JNK activation (Lisovsky M et al. 2002).

Moreover, the JNK pathway, which is the downstream signaling of Wnt-PCP pathway, has been shown a causal role in inducing apoptosis: one mechanism could be the upregulation of pro-apoptotic TNF superfamily members (TRAIL, FasL) at the transcription level; the other mechanism may be related to the inactivation of Bcl-2 by phosphorylation (Basu S et al. 1998; Shen HM et al. 2006).

In the canonical Wnt signaling, accumulated β-catenin can translocate to the nucleus and activate TCF/LEF to regulate the expression of cell proliferation-related genes. Transgenic mice expressing a stabilized form of β-catenin in T cell compartment showed increased number of $CD4^+CD8^+$ double-positive (DP) thymocytes than wildtype, and DP thymocytes protection against spontaneous and glucocorticoid-induced apoptosis by upregulating Bcl-xL levels transcriptionally (Xie H et al.2005). However, the same group found that the β-catenin transgenic mice had accelerated deletion of $V\beta8^+$ $CD4^+$ T cells than wildtype mice, which was associated with AICD by promoting Fas-induced T cell apoptosis through enhancing Fas promoter activity (Huang Z et al. 2008). These results indicate that β-catenin has distinct roles between in early developing T cells and in peripheral mature T cells. Moreover, in HCT116 colon cancer cells, stabilized β-catenin inhibited FOXO3a-induced cell apoptosis (Dhener M et al. 2008).

Axin, a component of β-catenin destruction complex, shows pro-apoptotic functions as a tumor repressor in cells. Exogenous Axin expression induced cytochrome c release, while knockdown of Axin attenuated UV-induced apoptosis *in vitro* (Li Q et al. 2007). Upon UV irradiation, Axin is found to translocate to the nucleus where it binds Daxx, a death-domain associated protein, to interact with p53, thereby inducing apoptosis by stimulating p53 transcription activity (Li Q et al. 2007; Lin SC et al. 2007). In

astrocytoma cases, the expression levels of Axin was inversely correlated to the progression of the tumor, and overexpression of Axin in astrocytoma cells promoted cell death while inhibited cell proliferation possibly through the p53-dependent pathway (Zhang LY et al. 2009).

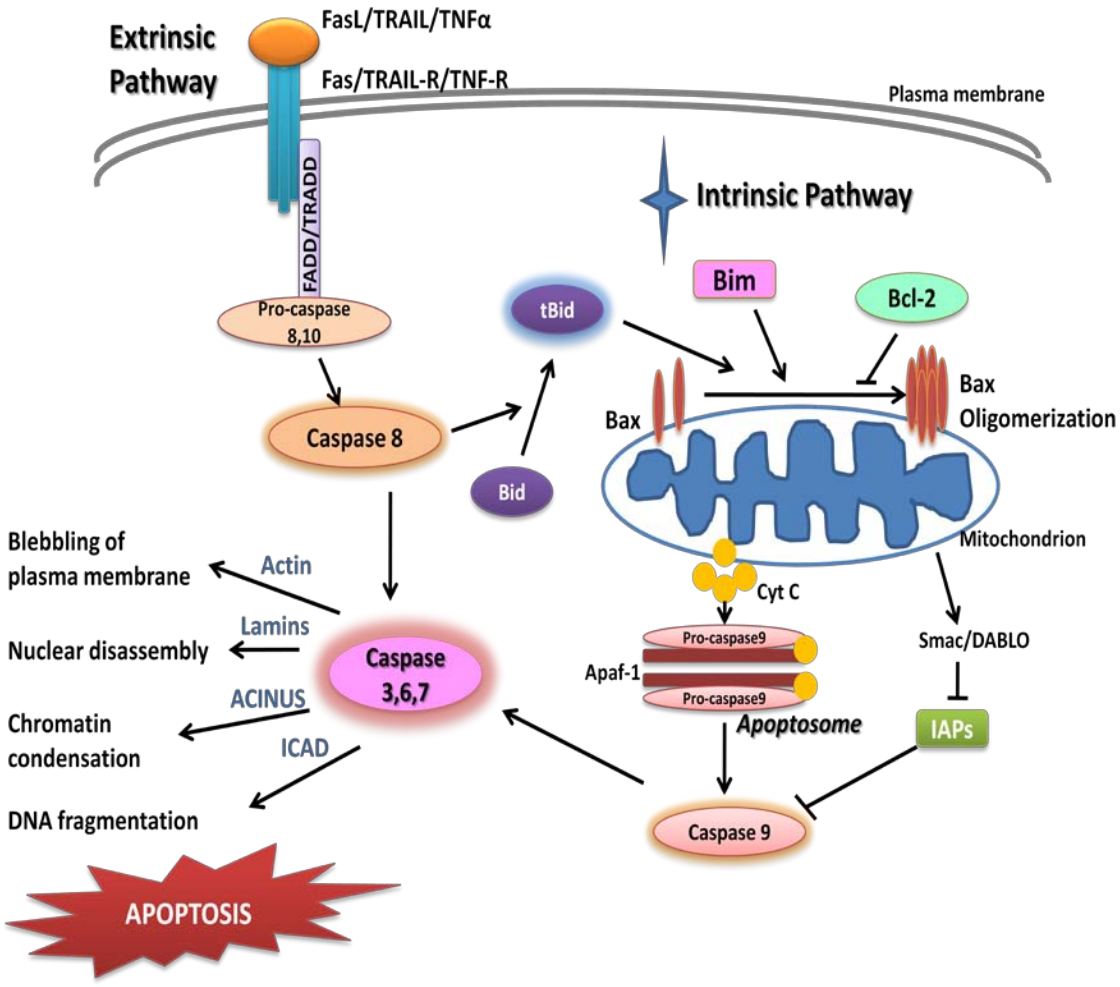


Figure 1.3 Schematic representation of the extrinsic and intrinsic apoptotic pathways. In the intrinsic apoptotic pathway, BH3-only proteins like Bim liberate Bax from Bcl-2 and promote Bax oligomerization and the formation of MOMP, resulting in the release of pro-apoptotic molecules like cytochrome c, Smac/DIABLO from the mitochondria. Cytochrome c binds and activates apoptotic protease-activating factor (Apaf)-1, forming the apoptosome complex with procaspase-9, which leads to the generation of active caspase-9. Meanwhile, Smac can inhibit inhibitor of apoptotic proteins (IAP) to further activate caspase-9, which in turn, induces the cleavage of executioner caspase-3, 6 and 7. In the extrinsic apoptotic pathway, the interaction between Fas ligand/TRAIL/TNF and

Fas/DR4, 5/TNFR leads to the recruitment of adapter proteins FADD and TRADD, which then bind procaspase-8, and subsequently caspase-8 is activated to trigger effector caspases either directly or through a BH3 only protein tBid. The activation of executioner caspases will cleave their substrates (actin, Lamins, ACINUS, ICAD) to induce morphological changes and DNA fragmentation in apoptotic cells (Blank M et al. 2006).

CHAPTER 2. CENTRAL HYPOTHESIS AND SPECIFIC AIMS

Wnt proteins are critical for development and adult homeostasis by controlling cell fate (Moon RT et al. 1997), and they can have either pro-apoptotic or anti-apoptotic functions in different cell systems (Almeida M et al. 2005; Lobov IB et al. 2005). Wnt13 is one of the major Wnt genes expressed in differentiated endothelial cells (Struewing IT et al. 2006). Three isoforms of human Wnt13 have been identified in endothelial cells with different subcellular localizations: secreted Wnt13A (localized in endoplasmic reticulum), mitochondrial Wnt13B and nuclear Wnt13C, which are generated through alternative promoter, alternative splicing, and alternative translational start sites (Struewing IT et al. 2006). Wnt13 nuclear forms showed an increased sensitivity to LPS or TNF induced apoptosis in primary endothelial cells (Struewing IT et al. 2006). However, it is unclear whether the expression Wnt13 isoforms undergo regulation and whether there are differential functions of Wnt13 isoforms depending on their subcellular localizations.

Central hypothesis: In differentiated endothelial cells, the expression of nuclear Wnt13C is regulated at translational levels during apoptosis; and nuclear Wnt13 isoforms favor apoptosis through regulating the activity or expression of apoptosis-related factors; also, distinct isoforms of Wnt13 may have differential effects on endothelial cell apoptosis and apoptosis-related factors.

Wnt13C and short form of Wnt13B encodes the same protein translated form AUG+74, but the expression of this protein encoded by Wnt13C was found out much

lower than those which are encoded by Wnt13B and M1L-Wnt13B (Struewing IT et al. 2006), suggesting a possible regulation during Wnt13C translation. During apoptosis, the general translational machinery via cap-dependent mechanism is inhibited. But, protein synthesis of some apoptotic-related factors is required to help cell fate decision of either self-recovery or suicide, so alternative mechanisms, such as internal ribosomal entry, are employed to initiate translation of some proteins, including anti-apoptotic XIAP, Bcl-2, as well as pro-apoptotic c-myc, Apaf-1, p53. Since nuclear Wnt13 forms increase the endothelial cell susceptibility to apoptotic stimuli, it is of interest to know if nuclear Wnt13C behaves like other pro-apoptotic or anti-apoptotic factors (c-myc and Bcl-2), which expression is maintained or induced under stress and regulated at translational level during apoptosis.

Aim 1: To study whether the expression of nuclear Wnt13 form is regulated at translational levels during endothelial cell apoptosis (Figure 2).

Cell apoptosis is believed to be a key player in embryonic development and cell homeostasis, and it can be tightly regulated at different levels (Blank M et al. 2007; Elmore S. 2007). Apoptosis is controlled by the balance of pro-apoptotic factors against anti-apoptotic factors, which serves as an indicator of cell susceptibility to apoptosis (Nakajima T et al. 2006). Indeed, apoptotic events take place in different subcellular organelles: the release of cytochrome c from the mitochondria, caspase cleavage in the cytoplasm, and DNA fragmentation or the transactivation of transcription factor p53 in the nucleus. Therefore, three Wnt13 isoforms may show differential effects on

endothelial cell susceptibility to apoptosis through regulating different apoptotic events in the organelles where they are located.

Also, the mechanism that increased endothelial cell apoptosis by nuclear Wnt13 has yet to be investigated. The Bcl-2 family includes pro-apoptotic members like Bax and Bim as well as anti-apoptotic members like Bcl-2, which balance functions as a crucial switch to determine whether the cell undergo apoptosis (Youle RJ et al. 2008); also, Bcl-2 members are involved in the Wnt-related apoptosis during development (Liang H et al. 2007). Akt signaling is a pro-survival pathway, which inactivation leads to cell apoptosis by activating pro-apoptotic factors such as caspase-9, Bcl-2 family member Bad, and FOXO factors (Jiang BH et al. 2008; Dillon RL et al. 2007). And Akt signaling not only cross-talks with Wnt signaling through GSK-3β, but it plays a critical role in Wnt cascade (Longo KA et al. 2002; Almedida M et al. 2005; Constantinou T et al. 2008; Naito AT et al. 2005). Therefore, it is necessary to study whether the nuclear Wnt13 forms increase apoptosis in endothelial cells through tipping the balance of Bcl-2 family to pro-apoptotic members, and the involvement of Akt signaling.

Aim 2: To study the differential effects of Wnt13 isoforms located in different subcellular organelles in endothelial cell apoptosis, and whether Wnt13 forms affect apoptosis though modulating the activity or expression of apoptotic effectors and regulators (Figure 2).

In Aim2, we found that nuclear Wnt13 forms increased endothelial cell sensitivity to apoptosis possibly through upregulating the activity/expression of caspases, and Bim

expression. FOXO transcription factors, which activation is inhibited by Akt survival signaling, target and transactivate some apoptotic-related genes like *caspase-3*, *Bim*, *FasL* as well as *TRAIL*, and can induce apoptosis when constitutively activated in certain cell types (Huang H et al. 2007). Additionally, FOXOs are also found to converge with Wnt-signaling (Essers MA et al. 2005). Therefore, FOXOs could be the upstream regulator to increase BAEC apoptosis by Wnt13 proteins. Moreover, stress resistance-related *SOD2* and *catalase* are also the target genes of FOXOs (Huang H et al. 2007), and activated FOXOs can bind Forkhead binding site in *SOD2* gene to promote *SOD2* gene transcription (Kops GJ et al. 2002), so the expression and transcription of *SOD2* and *catalase* may be another way to confirm FOXO activation since different post-translational modifications of FOXOs might be important for apoptotic genes and oxidative stress resistance genes. Therefore, to confirm the activation of FOXOs by Wnt13 proteins, it is interesting to know the effect of Wnt13 forms on the expression of oxidative stress resistance genes and *SOD2* transcriptional regulation.

Aim 3: To study whether Wnt13 forms have differential effects on the expression and activation of FOXOs that mediate the transcriptional regulation of FOXO target genes related to apoptosis and oxidative stress resistance (Figure 2).

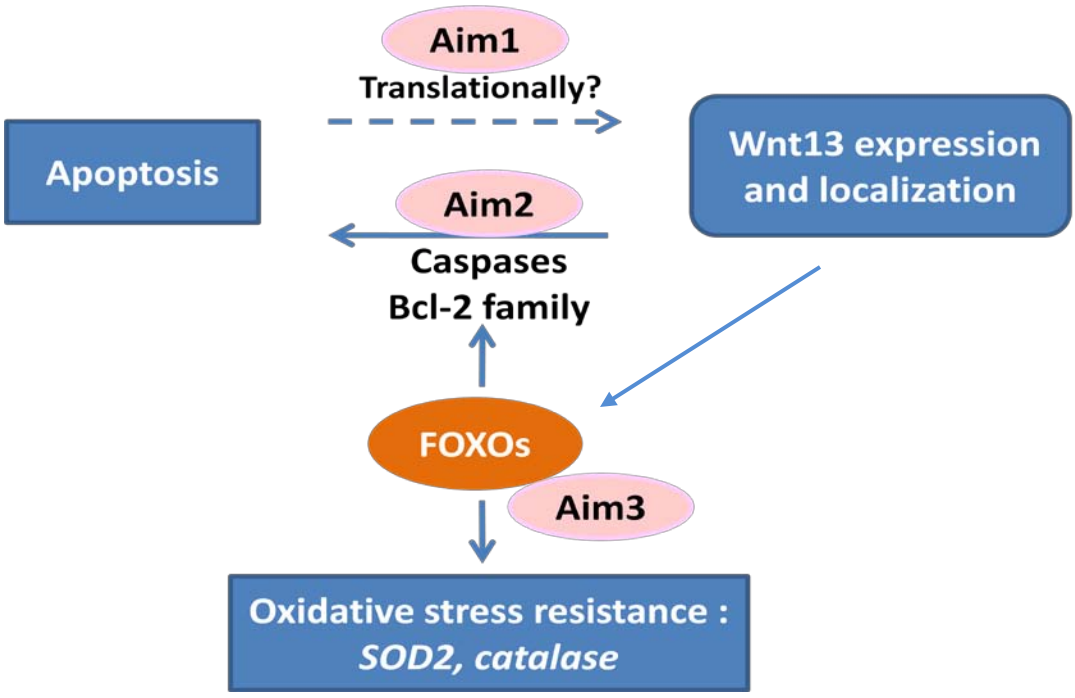


Figure 2 Specific aims in this dissertation. As some apoptotic factors are translationally regulated by cell apoptosis, the Aim1 is to study whether the expression of nuclear Wnt13 form is regulated at translational levels during endothelial cell apoptosis. Since caspases, Bcl-2 family members and FOXOs are crucial players in apoptosis, the Aim 2 is to study the differential effects of Wnt13 forms located in different subcellular organelles in endothelial cell apoptosis, and whether Wnt13 forms affect apoptosis through modulating expression/activation of apoptotisis-related factors. Moreover, FOXOs are transcription factors to transactivate pro-apoptotic factors such as Bim, and *SOD2* and *catalase* are also oxidative stress resistance-related FOXO target genes, so Aim3 is to study whether Wnt13 forms have differential effects on the expression and activation of FOXOs that mediate the transcriptional regulation of FOXO target genes related to apoptosis and oxidative stress resistance.

CHAPTER 3. GENERAL METHODS

3.1 Materials

Lipolysaccharide (LPS) and tumor necrosis factor (TNF) α were purchase form Calbiochem. The rabbit polyclonal antibodies against β-actin, D175 cleaved-caspase-3, and the secondary horseradish peroxidase (HRP) –conjugated antibodies were products from Cell Signaling Technology. The rabbit polyclonal anti-Flag tag antibodies were purchased from Cayman-Chemicals. Alexa-488 and Alexa-568 secondary antibodies as well as Mitotracker-633 deep red were products from Moleuclar Probes.

3.2 Cell culture

The bovine aortic endothelial cells (BAEC) were purchased from Cambrex, and maintained in 1 g/l glucose Dulbecco Modified Enriched medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37^{o}C, 5% CO_2.

3.3 Transient transfection

Cells were plated and grown to 70% confluency before transfection. Cell transient transfections were performed with ExGen 500 reagent (MBI Fermentas) according to the recommendations of the manufacturer. Briefly, for 1×10^5 cells, 1 µg plasmid DNA was diluted with 50 µl sterile 150 mM NaCl, and mixed with 50 µl diluted ExGen 500 reagent (1:9 in150 mM NaCl). The ExGen-DNA complex was then incubated at room

temperature for 15 minutes, and mixed with DMEM (no serum). After media change, cells were incubated with ExGen-DNA complex under 37^{o}C, and 2 hours later, the transfection complex was replaced by complete growth media.

3.4 Plasmid constructs

The Wnt13A-Flag, Wnt13B-Flag, Wnt13C-Flag, M1L-Wnt13B-Flag and M1L-Wnt13B-Flag expression constructs were previously described (Struewing IT et al. 2006).

3.5 RNA isolation and real-time PCR

3.5.1 RNA isolation

Total RNA was isolated using Trizol reagent (Invitrogen). Briefly, for a 6 well plate, cells in each well were added with 1ml Trizol after medium removal, and scrapped off the plate, followed by being transferred into a microcentrifuge tube. 200 µl Chloroform was then added to the tube, and mixed. After 10 minute incubation, the Trizol-$CHCl_3$ mixture was centrifuged at 12,000g for 10 minutes. The top layer was transferred to another tube, and mixed with 500 µl isopropanol prior to another 10 minute incubation and centrifuge. The RNA pellet was then washed by 500 µl 70% ethanol, followed by centrifugation for 5 minutes. After the supernatant was discarded, the pellet was left in room temperature until it became clear. Then 22 µl nuclease free water was added to dissolve the pellet. 1µl of RNA solution was used to measure the OD value and 260/280 ratio by spectrometer, and another 1µl of RNA was loaded to fresh 1% agarose gel for electrophoresis to check the RNA quality. The isolated RNA was either used for reverse transcription immediately or stored at -80^{o}C for later experiments.

3.5.2 Reverse Transcription

Total RNA (1μg) was firstly treated by DNase-I (Invitrogen) with reaction buffer for 15min at room temperature to remove any genomic DNA contaminations until 2 μl 25 mM EDTA was added to stop the reaction. After 10 minute incubation at 65 ^{o}C, The RNA-DNase I mixture was transferred on ice and added with 0.5 μg Oligo(dT) (Invitrogen), followed by another 10 minute incubation at 65 ^{o}C. The RNA mixture was then left on ice for at least 1 minute prior to being incubated with 1 μl (200U) SuperScript III reverse transcriptase (Invitrogen) as well as its reaction buffer (1 μl 0.1 M DTT, 0.6 μl RNaseOUT (Invitrogen), and 0.4 μl 25mM dNTP) at 50 ^{o}C for 1 hour, then inactivated by incubation at 70 ^{o}C for 15 minutes. Then the 20μl cDNA solution was diluted to 60 μl of total volume with Nuclear-Free Water and then stored in -80 ^{o}C.

3.5.3 Real-time PCR

The real-time PCR was performed in duplicates, with an equivalent of 16ng total RNA reaction and 10 pmoles of primers, using the SYBR-Green Kit (Applied Bio-systems) in ABI7000 apparatus (Applied Bio-systems). Each 25μl reaction also contained 1× SYBR-Green PCR reaction buffer, 3.0 μl 25mM $MgCl_2$ solution, 2.0 μl 12.5mM dNTP, 0.25μl 1U/μl Amperase UNG, 0.125μl 5U/μl AmpliTaq Gold. RPL30 was used as the internal standard gene. Real-time PCR conditions for all genes were as follows: 2 minutes at 50 ^{o}C; 10 minute at 95 ^{o}C; 40 cycles of 15 seconds at 95 ^{o}C and 1 minute at 60 ^{o}C. At the end of the runs, data were analyzed. Ct values obtained from the amplification curve of the gene of interest were normalized to that obtained form the amplification curve of RPL30, to correct for equal amounts of starting RNA.

3.6 Cell extracts and western blot analysis

For whole cell extracts, cells were harvested by centrifugation at 600g for 5 minutes after being scrapped off the plates. After washing by PBS buffer, each 1×10^5 cells were lysed with 125 µl 50mM HEPES, pH7.4, 0.1% CHAPS, 5mM DTT, and 2mM EDTA in the presence of protease and phosphatase cocktail inhibitors (Sigma). After freeze-thaw cycles, each 125 µl cell lysate was added with 25 µl 6× Laemmli buffer, and then boiled for 5 minutes. Subsequently, 25ul lysates from each sample were loaded on SDS-polyacrylamide gels (ranging from 8% to 14%, depending on the size of protein of interest) for electrophoresis, and proteins were then transferred onto Immobilon P membrane (Minipore). After blocking with 3% fat free milk (BioRad), the membranes were incubated with specific primary antibodies overnight, followed by washing with 3 time TBS-Tween buffer (10 minutes for each time). Subsequently, the membranes were incubated with the corresponding secondary antibodies conjugated to horseradish peroxidase (HRP) for 1-2 hours and also followed by the same wash for 3 times. Each membrane with Immuno-reactive proteins and conjugated HRP were detected using the mixture of 1.5 µl stable peroxide buffer and 1.5 µl Lumino enhancer buffer supplied by SuperSignal chemiluminescence (Pierce Chemical Co), and exposed for an appropriate time ranging from 1 second to 20 minutes. At last, the intensity of each band including loading control β-actin was quantified by densitometry with Scion software, and the expression level of each protein of interest was shown as the ratio of Density (protein of interest)/Density (β-actin).

3.7 Plasmid DNA purification

3.7.1 Minipreps

For small amount of plasmid DNA isolation, Wizard® *Plus* SV Minipreps DNA Purification Systems (Promega) were used. According to the manufacture's instruction, for each sample, 1ml bacterial culture was harvested by centrifugation for 5 minutes at highest speed (14×10^3 rpm) in a tabletop centrifuge. Supernatant was then poured, and 250µl Cell Resuspension Solution was added to the cell pellet, followed by thorough resuspension by pipetting. 10µl Alkaline Protease Solution was then added and mixed with cell pellet suspension by inverting the tube 4 times. Subsequently, 250µl of Cell Lysis Solution was added and mixed by inverting the tube 4 times, which was then incubated for 5 minutes. 350µl Neutralization Solution was added to the tube and mixed immediately by inverting the tube 4 times, followed by centrifuging the bacterial lysate at maximum speed for 10 minutes at room temperature. Afterwards, the cleared lysate was transferred to the prepared Spin Column by decanting, and then centrifuged at maximum speed for 1 minute at room temperature. The Spin Column was removed, and reinserted into the Collection Tube after flowthrough was discarded from the Collection Tube. The Spin Column was then added by 750 µl Column Wash Solution, and centrifuged at maximum speed for 1 minute at room temperature, with flowthrough discarded. The wash procedure was repeated using 250 µl Column Wash Solution, followed by centrifugation at maximum speed for 2 minutes. Then, the Spin Column was transferred to a new, sterile microcentrifuge tube, and plasmic DNA was eluted by adding 100 µl Nuclease-Free Water and subsequent centrifuging for 1minute.

3.7.2 Maxipreps

For large amount of plasmid DNA purification, UltraMobius^{TM} 200 plasmid Kits (Novagen) were used in our study, based on the manufacture's manuals. For each sample, 100ml bacterial culture was centrifuged at 6,000 rpm for 20 minutes (4^{o}C), and the pellet was then resuspended in 3ml Bacterial Resuspension Buffer supplemented with RNase A. After this, the pellet was added with 3ml Bacterial Lysis Buffer, which was then mixed by inverting the bottle, followed by 5 minute incubation at room temperature. 3ml of Mobius Neutralization Buffer was added to the lysate, which was then incubated on ice for 5 minutes, prior to centrifuging at 10,000 rpm for 20 minutes. The supernatant was filtered, supplemented with 1.5ml Detox Agent, and incubated on ice for 30 minutes. The supernatant mixture was then applied to the equilibrated Mobius 200 Column, which was subsequently washed with 10ml Mobius Wash Buffer, and eluted with 2.5ml Mobius Elution Buffer. The elution was added and mixed with 1.75ml isopropanol prior to the centrifugation at 15,000 rpm for 20 minutes. Then, the pellet was washed by 3ml 70% ethanol, followed by another centrifugation for 10 minutes. Once dried, the pellet was dissolved in 400ul TE buffer, and the OD value was measured by spectrometer before the storage of the purified DNA solution.

3.8 Immunofluorescence microscopy

BAECs were plated in LabTek slide chambers 24 hours prior to transfection. 40 hours after transfection, when required, BAECs were incubated with 100nM Mitotracker-633 deep red (Molecular Probes) in growth media for 1 hour to stain mitochondria, followed by cell washes 3 times with PBS supplemented with 1mM $CaCl_2$ and 1mM

$MgCl_2$. Then cells were fixed in 4% formaldehyde for 17 minutes prior to 3 time cell washes, and were permeabilized and blocked in PBS, 0.1% Triton X-100, 0.7% fish skin gelatin (Sigma) at 37^oC for 30 minutes. Fixed cells were then incubated sequentially with primary antibodies and Alexa-secondary antibodies for 2 hours each in the gelatin buffer, and each incubation was followed by 3 time cell wash using the gelatin buffer. Subsequently, cells in the chambers were finally washed with PBS ($CaCl_2$ and $MgCl_2$), and mounted with Vectashield H1200 (Vectro Laboratories Inc.) supplemented with 4', 6-diamidino-2-phenylindole (DAPI) to stain nuclei, and finally observed under Nikon ECLIPSE TE 2000-U.

3.9 Statistical analysis

All results are expressed as mean ± SEM. Analysis of variance (ANOVA) is valid if 1) all groups of observations represent random samples; 2) the population distributions must be approximately normal (Probability and statistics Ebook, Statistics Online Computational Resource, SOCR). Thus, for the values represented by the relative levels over PCR3 control, if PCR3 control was set as 1, which did not fall in normal distribution, One Way ANOVA analysis was not appropriate at this point; therefore, as suggested by stasticians, One Sample T-test (hypothesized value = 1) was used. Statistical significance was accepted at a value of $P<0.05$. However, if the values of PCR3 control follow normal distribution, One Way ANOVA analysis was used followed by Tukey's test for differences between groups.

3.10 Wnt13-Flag-expressing system

3.10.1 Transient transfection

After transient transfection, the expression of Wnt13-Flag isoforms was detected by immunoblotting. As shown in Figure 3.1, PCR3 was the vector control which did not express Wnt13-Flag proteins. Wnt13A-Flag displayed one single band of 41KD in size, and Wnt13B-Flag expressed a protein doublet because Wnt13B mRNA gave rise to a long form and a short form at protein levels due to alternative start codons, (AUG (+1) and AUG (+74) (Struewing IT et al. 2006). For Wnt13C-Flag, only one single protein equivalent to short form of Wnt13B was detected, and the expression level is very low. M1L-Wnt13B-Flag only encoded the short form of Wnt13B because the first codon AUG (+1) was mutated, and the expression was dramatically higher than that of Wnt13C, which will be stated in the Aim1. And M74L-Wnt13B exclusively expressed the long form of Wnt13B since AUG+74 was mutated. The expression pattern in Wnt13-Flag protein level during transient transfection were M1L-Wnt13B>Wnt13B>Wnt13A>M74L-Wnt13B>Wnt13C. Moreover, the protein level of Wnt13C was increased upon LPS treatment, which is also described in Aim1.

3.10.2 Stable transfection

We have tried to track the expression of Wnt13-Flag in stably-transfected BAECs by western blotting, but except Wnt13A, all the other forms of Wnt13 were almost undetectable (data not shown), suggesting that stable transfection has lower expression levels than transient transfection. Therefore, we employed immunofluorescence microscopy to determine the expression of Wnt13-Flag in stably-transfected BAECs. As

shown in Figure 3.2, the subcellular localization of Wnt13 isoforms in stable transfection was not distinguishable than that in transient transfection: Wnt13A-Flag was localized in ER; Wnt13B and M74L-Wnt13B were mitochondrial forms; Wnt13C and M1L-Wnt13B were localized in the nucleus. However, the expression pattern in stable transfection was found out different: Wnt13A>Wnt13C=M1L-Wnt13B>M74L-Wnt13B>Wnt13B (data not shown). And this difference in protein levels may underlie the difference in the effects of Wnt13 forms between stable transfection and transient transifection which is described in Aim2.

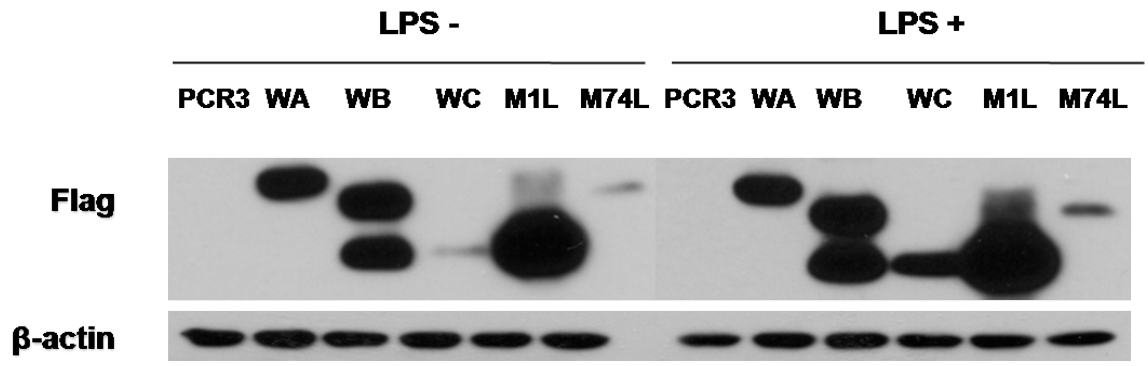


Figure 3.1 The expression patterns of Wnt13-Flag isoforms in transiently transfected BAECs with or without LPS treatment. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 24 hours followed by being treated with or without 100ng/ml LPS. 16 hours later, cells were harvested and lysed whole cell exacts were prepared and Flag-tag and β actin were analyzed by immunoblotting with specific antibodies.

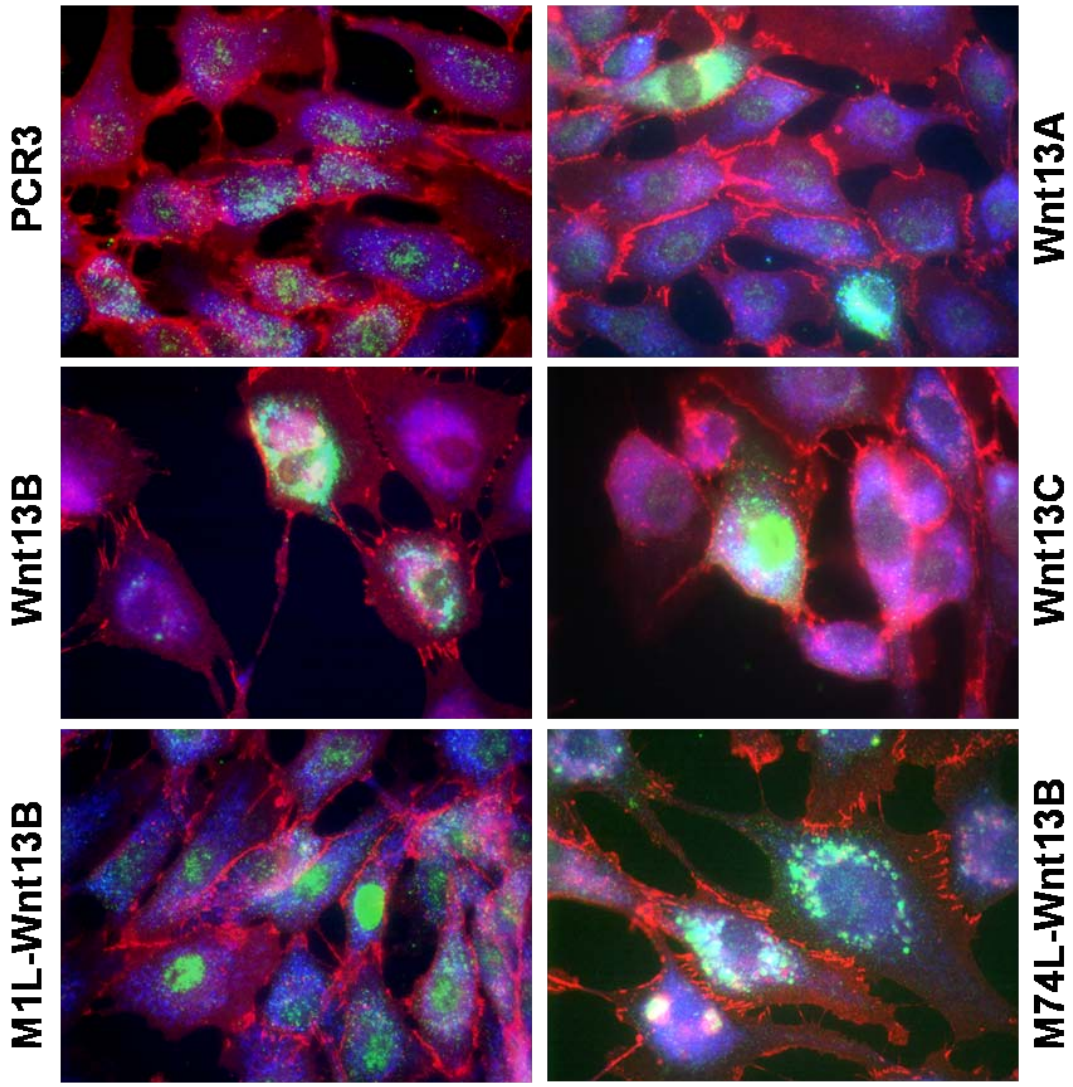


Figure 3.2 The expression patterns and subcellular localizations of Wnt13-Flag isoforms in stably transfected BAECs. BAECs with stably-expressed Wnt13-Flag constructs were incubated with growth media supplemented with Mitotracker-633 deep red for mitochondrial staining (blue), and then fixed with 4% formaldehyde, permeabilized in 0.1% Triton, and stained with rabbit polyclonal anti-Flag antibody (green) and mouse monoclonal β-catenin (red), followed by the incubation of Alexa goat anti-rabbit 488 and Alexa goat anti-mouse 568 antibodies.

CHAPTER 4. SPECIFIC AIM 1

To study the expression of nuclear Wnt13 forms is regulated at translational levels during endothelial cell apoptosis

4.1 Summary

Wnt proteins control cell proliferation, cell differentiation and cell fate during embryonic development and homeostasis in adulthood. Our laboratory has demonstrated the complexity of human Wnt13 gene in human cells, and the association of the nuclear Wnt13 forms with cell apoptosis, but the regulation of Wnt13 forms has yet to be defined. Wnt13B, Wnt13C and M1L-Wnt13B have been shown to encode the same protein translated form AUG+74, but the expression of this protein encoded by Wnt13C mRNA is found out much lower than those who encoded by the mRNAs of Wnt13B and M1L-Wnt13B (Struewing IT et al. 2006), suggesting a possible translational regulation for Wnt13C. In our study, Wnt13C expression was increased in response to stress and apoptosis-inducers (including MG132), and appeared to be correlated with caspase-3 cleavage. This regulation did not seem to occur at the transcriptional level since there was no significant increase at mRNA level upon the treatment of MG132. And in BAEC, the insertion of Myc tag at the first AUG in Wnt13C mRNA not only inhibited the expression of exogenous Wnt13C at basal level, but also totally abolished the increase of Wnt13C expression by MG132 treatment, which indicates RNA sequences or structures are critical for Wnt13C expression. Therefore, our data suggest that nuclear Wnt13C can be regulated during apoptosis more likely at the translational levels than the transcriptional levels, and since the nuclear Wnt13 forms were reported to increase EC

sensivity to apoptosis (Struewing IT et al. 2006), this regulation may underlie a possible association of the nuclear Wnt13 forms with apoptosis in BAECs.

4.2 Introduction

Wnt proteins form a large family of cysteine-rich glycoproteins which expression is tightly regulated in a spatial and temporal fashion during development and adult homeostasis. So far there are 19 different Wnt members in mammals, and they function critically in embryonic development, organogenesis and later-on self-renewal in adult tissues by controlling cell fate, including cell proliferation, differentiation, and apoptosis (Clevers H. 2006; Almeida M et al. 2005).

The complexity of human Wnt13 gene has been displayed in various human cell types with three isoforms identified: secreted Wnt13A, mitochondrial Wnt13B and nucleus Wnt13C, which differ in their N-terminal sequences and generated through alternative promoter, alternative splicing and alternative start codons (Struewing IT et al. 2006; Bunaciu RP et al. 2008). Moreover, the Wnt13 nuclear forms showed an increased sensitivity to LPS- or TNF- induced apoptosis in primary endothelial cells (Struewing IT et al. 2006), indicating the function of the Wnt13 nuclear forms is associated with apoptosis. The 5'-leader sequences of Wnt13B and Wnt13C mRNAs are rather long, featuring highly stable secondary structures based on M-fold analysis, which are the characteristics suggesting possible translational regulation (Tang T et al. 2008).

Increasing evidence has suggested that translational regulation is a crucial mechanism to allow the cell to alter its phenotype in response to different environmental changes, and the expression of a number of either apoptosis-related factors or growth

factors (FGF2 and VEGF) via translational mechanisms plays a significant role in the regulation of their functions (apoptosis or proliferation) (Graber TE et al. 2007; Willis AE. 1999). During cell stresses, the global translational machinery is compromised, and the translation of most mRNAs is thereby inhibited. Paradoxically, the synthesis of several proteins is still needed to mount an appropriate response to apoptosis by increasing either death signals or survival signals to help the cell make decision to die or not to die (Graber TE et al. 2007). So under stress, while the global rate of translation by classical cap-dependent mechanism is reduced, the mRNAs of some proteins are not treated the same way: alternative translational mechanisms are used to maintain the synthesis of these proteins. For example, during apoptosis, pro-apototic proteins c-myc (Adachi S et al. 2000), as well as anti-apoptotic proteins Bcl-2 (Sherrill KW et al. 2004) maintain their protein synthesis by employing a cap-independent mechanism called internal ribosomal entry, which involves specific RNA secondary structures able to recruit IRES trans-acting factors (ITAFs) that initiate translation from the alternative AUG site (Spriggs KA et al.2005; Marash L et al. 2005). Besides IRES, there are some other mechanisms also responsible for the process of translational control, such as ribosomal shunting, leaky scanning and re-initiation through small upstream open reading frames (ORFs) (Graber TE et al. 2007; Kozak M. 2003).

Therefore, in this study, we aim to investigate whether nuclear Wnt13 forms behave like other apoptotic factors like c-myc or Bcl-2, which undergo regulation at translational levels during apoptosis.

4.3 Materials and methods:

4.3.1 Materials

ALLN, MG132, LY294002 and tunicamycin were from Sigma. The specific proteasome inhibitors epoxomycin and eponomycin were kind gifts from Dr. Kyung-Bo Kim (University of Kentucky). The monoclonal anti-Myc antibody was purchased from Cell Signaling Technology.

4.3.2 Cell culture and transfection

Please refer to 3.2 and 3.3.

4.3.3 Myc-Wnt13C-Flag plasmid constructs:

To obtain the pCR3.1-Myc-Wnt13C construct, Myc-tag was inserted into the start codon AUG of Wnt13C by PCR using the primers 5'-CCACCATGGGCGAACAAAAACTCATCTCAGAAGAGGATCTGGTGTTGGATGGCCTTG-3', and 5'-TCACTTATCGTCGTCATCCTTGTAATCTGCGGTCTGGTCCAGCCAC-3' (Tang T et al. 2008). To obtain the pCR3.1-Myc-Wnt13C-Flag construct, as shown in Figure 4.1, pCR3.1- Myc-Wnt13C construct and pCR3.1-Wnt13B-Flag were digested by BamH1 (Invitrogen) at 37^{o}C for 2 hours, and the products were loaded to 1% agarose gel. The digest product from pCR3.1-Wnt13B-Flag was specifically treated with 1µl Calf Intestinal Alkaline phosphatase (CIAP, Invitrogen) for 5 minutes to dephosphorylate linerized vector DNA prior to gel running. After electrophoreses, a 300bp fragment from pCR3.1-Myc-Wnt13C and a 6kb fragment from pCR3.1-Wnt13B-Flag were expected

and extracted from the agarose gel using QIAGEN Gel Extract Kit. The two DNA fragments were then ligated using T4 ligase (Invitrogen) at 8-12 ^{o}C overnight, followed by transformation, which were subsequently spread evenly to a pre-warmed LB plate and incubated at 37^{o}C. After 18 hours, colonies were seen on the plate, and each of them was then picked gently and mixed with 3ml LB broth in each tube. After incubation for 6 hours, minipreps were performed for each colony to purify DNA, and 10µl of each purified DNA was digested by Pvu II (Invitrogen) to test the orientation during ligation. The DNA miniprep with right orientation was then sent to SegWright for sequencing, and maxiprep was performed to purify amplified pCR3.1-Myc-Wnt13C-Flag plasmid DNA, which was stored at -20 ^{o}C.

4.3.4 RNA isolation and real-time PCR

Please refer 3.5.

4.3.5 Cell extracts and western blot analysis

Pleases refer 3.6.

4.3.6 Statistical analysis

All results are expressed as mean ± SEM. The values were represented by the relative levels over PCR3 control (set as 1), and One Sample T-test (hypothesized value = 1) was used. Statistical significance was accepted at a value of $P<0.05$.

4.4 Results

4.4.1 Short form of Wnt13B, Wnt13C and M1L-Wnt13B express differently when transfected into BAEC

As described previously, Wnt13B and C mRNAs (Figure 1.2B) contain two translational start sites: AUG (+1) and AUG (+74), so Wnt13B mRNA encodes a long form and a short form (Figure 3.1). M1L-Wnt13B, where Met1 is replaced by leucine1, only gives rise to the short Wnt13B form (Figure 3.1), which is localized in the nucleus. However, for Wnt13C mRNA, the absence of all 71 nucleotides in exon 2 leads to a change in the open reading frame of exon 4 and formation of a stop codon, so the translation from AUG (+1) and AUG (+74) gives rise to a short peptide and the short Wnt13B form, respectively. But only the short Wnt13B was detected by immunoblotting in Wnt13C-transfected BAEC (Figure 3.1). Also, both Wnt13C and M1L-Wnt13B are localized in the nucleus when transfected into BAEC, further indicating Wnt13C and M1L-Wnt13B are equivalent to the short form of Wnt13B (Struewing IT et al. 2006). Nonetheless, in normal cell culture conditions, the exogenous expression of Wnt13C was always found out much lower than short protein form of Wnt13B or M1L-WntB (>10 folds) in primary BAEC cells (Figure 3.1).

4.4.2 The expression of Wnt13C increased in response to apoptotic inducers

Next, we tried to determine that whether the expression of Wnt13C can be also regulated during apoptosis in apoptosis-sensitive BAEC cells. Tunicamycin, an endoplasmic reticulum (ER) stress inducer (He L et al. 2009), and LY294002, a PI3 Kinase inhibitor, together with pro-inflammatory factors (LPS and TNFα) as well as

proteasome inhibitors (MG132, ALLN, epoxomycin and eponomycin) can induce apoptosis in various cell types. However, in our experimental conditions, BAECs were sensitive to apoptosis induced by 100ng/ml LPS, 10ng/ml TNFα, 1μM MG132, 1 μM epoxomycin and 1 μM eponomycin, but insensitive to 2.5 μg/ml Tunicamycin or 10 μM LY294002(LY). As shown in Figure 4.2A, Tunicamycin and LY294002 did not induce obvious apoptosis in BAEC as there was no or very little caspase-3 cleavage, and in this case, Wnt13C expression was not significantly changed. In contrast, BAEC cells underwent apoptosis upon the treatment of inflammatory agents like TNFα, LPS, as well as proteasome inhibitors ALLN, MG132, epoxomcin and eponomycin, and at the same time, the expression of Wnt13C was shown to be increased with these stimuli. Therefore, even endothelial cell apoptosis was induced at different levels and via different mechanisms, all the stimuli inducing apoptosis can increase the expression of Wnt13C in BAEC.

4.4.3 The regulation of Wnt13C by apoptosis may be at translational levels

The increased expression of Wnt13C upon the treatment of apoptotic stimuli could result from either transcriptional regulation or translational regulation of Wnt13C. Thereby, we performed real-time analysis and found that the mRNA levels of exogenous Wnt13C-Flag were increased by 47% after MG132 treatment (see Figure 4.2B), which can not explain the 4 fold-increase of protein expression by MG132, suggesting that the regulation of Wnt13C is not mainly due to transcriptional levels.

Furthermore, in BAEC cells, modification of AUG (+1) by the insertion of a myc-tag sequence in Wnt13C not only led to a total absence of Wnt13C expression from the

downstream AUG (+74) at basal levels, but also abolished the increase of Wnt13 expression induced by MG132 treatment. This phenomenon implies that the mRNA structures of Wnt13C, especially the sequences surrounding AUG (+1) is critical for the regulation of Wnt13C by MG132, confirming that the increased expression of Wnt13C by MG132 is more likely at translational levels than transcriptional levels. In contrast, in HEK293 cells, the insertion of myc-tag at AUG (+1) did not change the expression pattern of Wnt13C, indicating this regulation is cell type-specific (see Figure 4.3A). We also tried to track the small peptide translated from AUG (+1) of Myc-Wnt13C-Flag by using specific Myc antibody to see whether Wnt13C AUG (+1) was also regulated translationally. Unfortunately, even when 20% Acralymide gel was used, the Myc-tagged small peptide was undetectable (Figure 4.3B).

4.5 Discussion

Translational control is one of the critical regulatory mechanisms in eukaryotic gene expression, which allows the cell to respond rapidly to environmental changes (Graber TE et al. 2007; Willis AE. 1999). The majority of the mRNAs able to undergo translational regulation, especially those who encode growth factors and oncoproteins, carries long 5' untranslated regions (UTRs) with complex secondary structure that impedes the scanning during translational initiation, and such structure may harbor internal ribosomal entry site or upstream open reading frames (Willis AE. 1999; Pickering BM et al. 2005). Wnt13 proteins also function in controlling stem cell growth during development and are associated with human cancers. Also, like those mRNAs of growth factors, the 5'-UTRs of Wnt13B and Wnt13C are very long (120 nucleotides

before AUG+1, and 340 or 229 nucleotides before AUG+74 in Wnt13B or Wnt13C mRNAs, respectively), and highly structured with high free energies (-88~-104 kcal/mole for Wnt13B mRNA, and -70~-75 kcal/mole in Wnt13C mRNA) (Tang T et al.2008), which gives the evidence for sendonary structure to support possible translational regulation in the mRNAs of Wnt13B or Wnt13C (Willis AE. 1999).

Wnt13C and M1L-Wnt13B have been shown to encode the same protein (the short form of Wnt13B) which is translated from AUG+74 in the mRNA (Struewing IT et al. 2006). In this study, we found that the exogenous expression pattern of this protein in regular cell culture condition differs a lot: M1L-Wnt13B>short form of Wnt13B>Wnt13C (Figure 3.1). However, the exogenous mRNA levels between Wnt13B and Wnt13C lack the significant difference, suggesting that the translation of Wnt13C is not as efficient as that of Wnt13B or M1L-Wnt13B.

Our results also show that the expression of Wnt13C is increased during endothelial cell apoptosis triggered by a variety of stimuli. The protein expression of Wnt13C is correlated with the level of cleaved caspase-3, and all the proteasome inhibitors are able to induce higher levels of Wnt13C than TNFα and LPS, suggesting that increased protein stability by proteasome inhibitors may potentiate the increasing effect on Wnt13C-Flag expression by apoptosis-inducers. Subsequently, we also show that the slight increase of exogenous Wnt13C at mRNA levels after MG132 treatment, unable to fully explain the 4 fold increase of Wnt13C-Flag at protein levels, further indicating that the positive regulation of exogenous Wnt13C expression by stimuli may be more possibly at translational levels than transcriptional levels.

Moreover, as shown in Figure 4.3, in BAEC cells, the insertion of myc tag at AUG (+1) in the Wnt13C mRNA totally abolishes the exogenous protein expression of Wnt13C, even under the treatment of MG132, indicating that Wnt13C expression is greatly dependent on the RNA sequences and/or structures, especially surrounding AUG (+1). Indeed, our laboratory also showed that the deletion of the first 12 amino acids with a start codon inserted in Wnt13B mRNAs resulted in almost undetectable expression of both the long form translated from AUG (+12) and the short form translated from AUG (+74), while the deletion of the first 17 amino acids with a start codon inserted led to more expression of the long form than the short form (Tang T et al. 2008), which confirms that the RNA sequences and/or structures surrounding AUG+1 are crucial for the translation initiation at AUG (+74) for the mRNAs of both Wnt13B and Wnt13C. And our data showed that the translational regulation of Wnt13C occurred in BAEC cells, but not in HEK293 cells. The possible explanation is that HEK293 cells are immortalized transformed cells, which may determine distinct response of Wnt13C to undergo translational regulation by apoptosis than primary cells.

Since both c-myc and Bcl-2 have shown to be regulated via IRES-mediated translation, the presence of IRES in Wnt13C mRNA was firstly suspected to explain the regulation mechanism of Wnt13C. However, using dicistronic Renilla –firefly (FL) luciferase assay, the 5'-UTR of Wnt13C does not exhibit an IRES activity with or without the treatment of MG132 (Tang T et al. 2008).

As the presence of IRES in Wnt13C mRNA is excluded, upstream opening reading frame (uORF) becomes a possible mechanism for translational regulation of Wnt13C. In some mRNAs, there exist a number of AUG codons at the upstream of the

main open reading frame (ORF), which sequester ribosome from the AUG which precedes the main ORF, resulting in the general decrease of the initiation efficiency of translation (Meijer HA et al. 2002). For example, Bcl-2 contains a small ORF located from -119 to -84, which is well conserved between mouse, rat, chicken and humans. Deletion or mutation of this uORF from chloramphenicol acetyltransferase (CAT) reporter gene resulted in a significant increase in CAT activity in vitro; conversely, positioning this uORF into the reporter gene led to a remarkable inhibition of CAT protein production without decreasing CAT mRNA, which indicates that the uORF located within the 5'UTR of the bcl-2 gene is important and sufficient for translational regulation of bcl-2 gene (Harigai M et al. 1996).

Indeed, it has been demonstrated by our laboratory that the insertion of 5' leader sequences of Wnt13C into RL reporter lead to a 95% and 85% inhibition of translation activity in BAEC cells and HEK293 cells, respectively (Tang T et al. 2008). Furthermore, two uORFs were found in Wnt13C-leader sequences, and the expression of Wnt13C-Flag increased with the deletion of upstream AUG or CUG and the mutation of AUG (+1), indicating that two upstream ORFs are responsible for the reduced translational efficiency in Wnt13C. Also, the 5' leader sequences of Wnt13B contain only one uORF, which explains the higher expression of Wnt13B encoded from AUG+74 than Wnt13C.

In conclusion, our data demonstrated that nuclear Wnt13C undergoes regulation at translational levels during apoptosis induced by a variety of stress stimuli. Considering that nuclear Wnt13 forms favor apoptosis in endothelial cells, the upregulation of Wnt13C forms during apoptosis may potentiate the progression of apoptosis in endothelial cells, which forms a positive feedback to facilitate the completion of

apoptosis. And further studies in our laboratory showed that unlike some other apoptotic factors like c-myc and Bcl-2, the translational regulation of Wnt13C mRNA is not via IRES mechanism, but more likely attributed by the presence of upstream open reading frames.

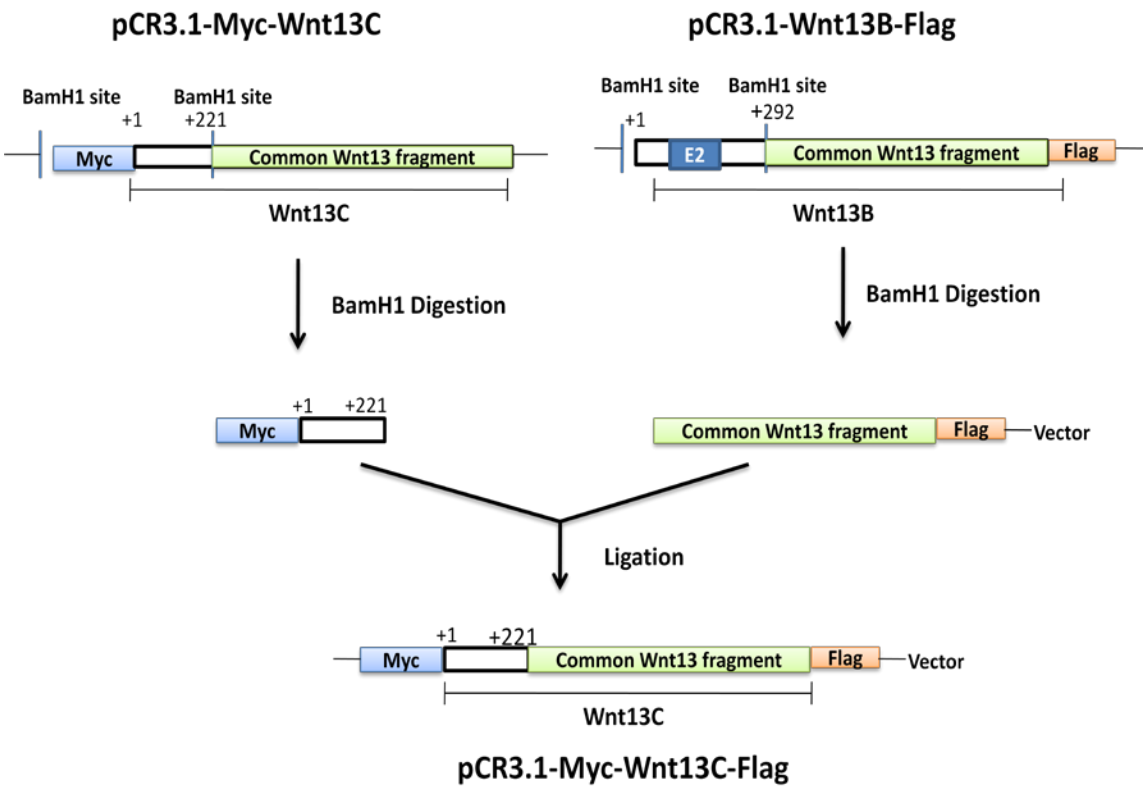
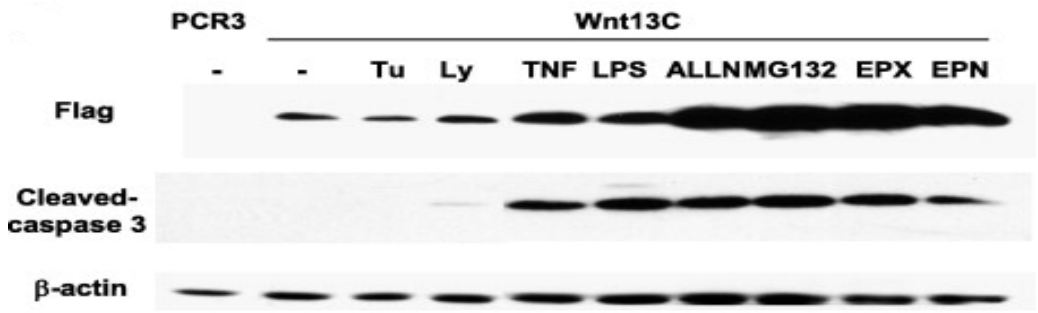


Figure 4.1 Schematic representation of the process to obtain the pCR3.1-Myc-Wnt13C-Flag construct. For mRNA sequence, Wnt13B has an extra exon2 (E2) compared to Wnt13C. pCR3.1- Myc-Wnt13C construct and pCR3.1-Wnt13B-Flag were digested by BamH1 (Invitrogen) at 37^{o}C for 2 hours, and the products were loaded to 1% agarose gel. After electrophoreses, a 300bp fragment from pCR3.1-Myc-Wnt13C and a 6kb fragment from pCR3.1-Wnt13B-Flag were extracted from the agarose gel using QIAGEN Gel Extract Kit. The two DNA fragments were then ligated using T4 ligase, followed by transformation to E coli, and the expressed pCR3.1-Myc-Wnt13C-Flag was purified by minipreps. After orientation check and sequencing check, the plasmid DNA was amplified by maxipreps.

A)



B)

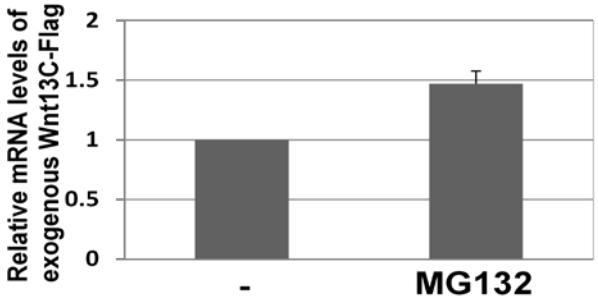
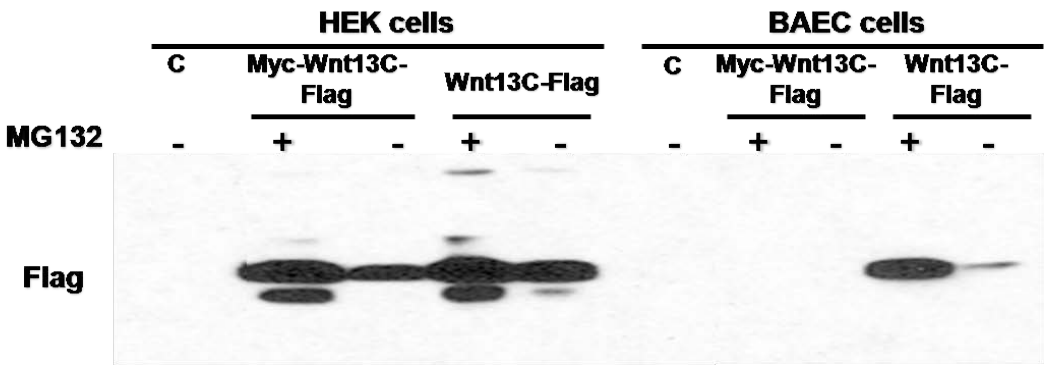


Figure 4.2 Wnt13C expression is increased in response to apoptotic stimuli. A) BAEC were transiently transfected with the Wnt13C-Flag construct for 24 hours, followed by being treated with 100ng/ml LPS, 10ng/ml TNFα, 2.5 μg/ml Tunicamycin (Tu), 10 μM LY294002(LY), 1μM MG132, 1 μM epoxomycin (EPX) and 1 μM eponomycin (EPN). 16 hours later, whole cell exacts were prepared and Wnt13C-Flag proteins, cleaved caspase-3 and β actin were analyzed by immunoblotting with specific antibodies. B) The mRNA levels of exogenous Wnt13C-Flag in BAEC with or without the treatment of 1μM MG132 were determined using real-time PCR with RPL30 as internal control. The relative mRNA levels after normalization are represented in the graph (mean±SEM, n=2 independent transfection experiments).

A)



B)

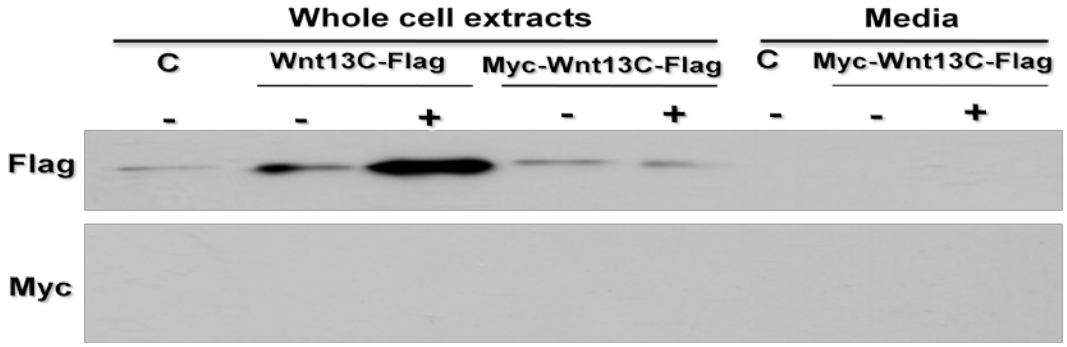


Figure 4.3 The insertion of Myc-tag into 5'-UTR of Wnt13C-Flag abolished the translation of Wnt13C in BAECs rather than in HEK293 cells. A) Wnt13C-Flag or Myc-Wnt13C-Flag were transfected into BAEC cells or HEK293 cells for 24hrs, followed by 1μM MG132 treatment for 16 hours, and whole cell extracts were prepared for subsequent immunoblotting analysis with anti-Flag antibody. B) Wnt13C-Flag or Myc-Wnt13C-Flag were transfected into BAEC cells for 24hrs, followed by 1μM MG132 treatment for 16 hours. Media were harvested and whole cell extracts were prepared for subsequent immunoblotting analysis with anti-Flag and anti-Myc antibodies.

CHAPTER 5. SPECIFIC AIM 2

To study the differential effects of Wnt13 isoforms located in different subcellular organelles in endothelial cell apoptosis, and whether Wnt13 forms affect apoptosis though modulating the activity or expression of apoptotic effectors and regulators.

5.1 Summary

Wnt proteins control cell proliferation, cell differentiation and cell fate during development and postnatal homeostasis. Our laboratory has illustrated the complexity of human Wnt13 gene, with three isoforms (secreted Wnt13A, mitochondrial Wnt13B and nuclear Wnt13C) encoded in human cells, and the association of nuclear Wnt13 forms with cell apoptosis. Also, nuclear Wnt13C was shown to be increased at the translational level during apoptosis induced by various stimuli including LPS. However, it is still unclear 1) whether Wnt13 forms exert different effects on cell apoptosis in BAECs due to their different subcellular localizations of Wnt13 isoforms; 2) what are the mechanisms underlying the increased apoptosis by nuclear Wnt13 forms. In this study, we found that 1) in all the apoptotic assays, the activity of Wnt13 forms in increasing EC sensitivity to apoptosis in BAEC were different: nuclear (robust) > mitochondrial (moderate) > secreted forms (weak); 2) Wnt13 forms affected executioner caspase-3/7, but not the caspase-8 in the extrinsic pathway, nor the inflammatory caspase-4/5: nuclear Wnt13 forms increased the expression of caspase-3/7 both at basal levels and after LPS treatment, followed by an increase in caspase-3/7 cleavage induced by LPS; 3) Wnt13

forms did not change the phosphorylation/activity of Akt and GSK, and showed a mild effect on ROS production; 4) exogenous Wnt13 forms upregulated the pro-apoptotic Bcl-2 family member Bim expression without changing Bax/Bcl-2 ratio; 5) Wnt13 forms did not significantly increase apoptotic factors (TRAIL, DR5, and caspase-8) in the extrinsic apoptotic pathway. To sum up, our data suggest that Wnt13 forms increase apoptosis in BAEC with different strengths: nuclear > mitochondrial > secreted forms, and Wnt13 forms increase caspase activation though upregulating the expression of caspases and Bim protein, without affecting the extrinsic apoptotic pathway, the Akt-GSK signaling and ROS production.

5.2 Introduction

Wnt proteins constitue a large family of cysteine-rich glycoproteins whose expression is tightly regulated in a spatial and temporal pattern during development. They bind specifically to frizzled receptors (Fz) to activate three different signaling pathways: 1) the Wnt/β-catenin/TCF pathway resulting in cell proliferation and cell survival, 2) the Wnt/planar cell polarity (PCP) pathway involved in cytoskeleton regulation and tissue morphogenesis and 3) the Wnt/Ca^{2+} dependent pathway leading to changes in cell migration (Fodde R et al.2007). Due to the crucial roles in organism development and postnatal homeostasis, Wnts and Wnt signaling are highly related to diseases, such as cancer, Alzheimer's disease, and metabolic syndrome (Clevers H. 2006). Wnt proteins are crucial for development and tissue homeostasis by controlling cell fate including cell proliferation, differentiation, and especially apoptosis (Moon RT et al. 1997). Generally,

Wnt proteins show anti-apoptotic action in transformed cells, but they display pro-apoptotic functions in primary cells, which details have been stated in 1.3.5.1.

So far, 19 different Wnt family members displaying a conserved pattern of 21-23 cysteine residues have been identified in mammals (Clevers H. 2006). Wnt13, also called Wnt2b, was shown to be critical for embryogenesis or organogensis, and to be related to some human diseases. We have shown the complexity of human Wnt13 gene expression from human cells with the identification of three Wnt13 isoforms: the secreted Wnt13A, mitochondrial Wnt13B, and nuclear Wnt13C forms, which were generated through alternative promoter, alternative splicing site and alternative start codon (Struewing IT et al.2006). Also, our laboratory showed that Wnt13 nuclear forms had an increased sensitivity to TNF-induced apoptosis in primary endothelial cells (Struewing IT et al.2006), and nuclear Wnt13C underwent translational regulation during apoptosis (Tang T et al. 2008). Indeed, apoptotic events take place in different subcellular organelles: the release of cytochrome c from the mitochondria, caspase cleavage in the cytoplasm, and DNA fragmentation or the transactivation of transcription factor p53 in the nucleus. Therefore, we are wondering whether the three Wnt13 isoforms have differential effects on endothelial cell apoptosis through regulating different apoptotic events in the organelles where they are located.

During apoptosis, caspase activation is one of the biochemical hallmarks. Caspase-3, 6, 7 are the executioner caspases which cleave proteins, including cytoskeletal proteins and nucleases, leading to the characteristics DNA breakdown and morphological modifications (Saraste A et al. 2000). Caspase-3 is thought to be the most important effector capases. Caspase-3, activated by caspase-8, caspase-9, or capase-10, has the

substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleaves a number of different proteins, such as poly (ADP-ribose) polymerase-1 (PARP-1), DNA-dependent protein kinase, which are important for apoptosis initiation (Villa P et al. 1997; Nicholson DW et al. 1995). Also, caspase-3 preferentially activates caspase activated DNase (CAD) and ACINUS to induce DNA degradation and chromatin condensation respectively, and also cause cytoskeletal modifications and the formation of apoptotic bodies (Elmore S. 2007).

The regulation in caspase expression at transcription levels or translational levels is important for caspase processing and activity (Eearnshaw WC et al. 1999). Human U937 cells treated by γ-interferon which can induce caspase expression showed enhanced susceptibility to apoptosis triggered by gamma-irradiation or antitumor agents (Tamura T et al. 1996), which is important for U937 cell differentiation to monocytes/macrophages. And based on the study from heterozygous and knockout mice of caspase-3, the expression of caspase-3 correlated to the sensitivity of T cells to apoptosis induced by etoposide (Sabbagh L et al. 2004), further indicating the significance of caspase-3 expression in apoptosis.

The activation of caspases is in particular regulated by Bcl2 family, which can be subdivided into three groups: the pro-apoptotic Bax and Bak, the anti-apoptotic family members Bcl-xl, Bcl-w, Mcl-1 and A1A, and the pro-apoptotic BH3-only proteins Bim, Bad, Bid (Youle RJ et al. 2008). Bax and Bak are major pro-apoptotic members in Bcl-2 family, which oligomerization will form the channels or pores at mitochondrial innner membrane, leading to the release of cytochrome c and initiation of downstream apoptotic events. However, Bcl-2 and Bcl-xL are major anti-apoptotic members, which form

heterodimer with Bax or Bak and inhibit their apoptosis-promoting function (Lomonosova E et al. 2008). The status of this pro-apoptotic/anti-apoptotic balance in Bcl-2 family not only governs whether the cell is committed to cell death, but it also determines the ability of the cell to respond to an apoptotic signal (Perlman H et al. 1999). The Bax/Bcl-2 rheostat has been shown to be critical for the sensitivity of human melanoma cells to cell death induced by Fas and ceramide (Raisova M et al. 2001); estrogen enhanced Bax/Bcl-2 ratio and induced apoptosis both in ovariectomized rats and cultured anterior pituitary cells (Zaldivar V et al. 2009). Moreover, the Bax/Bcl-2 ratio was found to accurately predict the clinical response and outcome of patients with acute myeloid leukemia because patients with higher Bax/Bcl-2 ratio had significantly longer overall survival and disease free survival (Del Poeta G et al. 2002). So Bax/bcl-2 ratio has been used as an index to roughly predict the susceptibility of the cell to apoptosis.

In addition, BH3-only proteins such as Bim can promote pro-apoptotic signals through either directly activating Bax, or inhibiting anti-apoptotic Bcl-2, resulting in Bax oligomerization (Danial NN. 2007). Bim-deficiency mice had abnormally high numbers of lymphoid and myeloid cells; and older Bim-deficient mice develop splenomegaly, lymphadenopathy, and autoimmune kidney disease, indicating that Bim is critical for apoptosis and homeostasis in the lymphoid and myeloid compartments (Bouillet P et al. 1999). Overexpression of Bim induced a remarkable increase of apoptosis in NIH 3T3 fibroblasts (Marani M et al. 2002) and in Hela cells (Herrant M er al. 2004). Therefore, Bim serves as a key initiator of the intrinsic pathway of apoptosis, and the regulation of Bim expression and activity can tightly affect the downstream apoptotic events.

Bcl-2 family is the hub to connect the intrinsic apoptotic pathway with the extrinsic apoptotic pathway (Youle RJ et al. 2008). In endothelial cells, LPS-induced apoptosis involves the extrinsic pathway (Bannerman DD et al. 2003). Generally, the extrinsic apoptotic pathway is dependent on transmembrane death receptors binding extracellular ligands, (for example, DR5/TRAIL is one of the death receptor/ligand pairs), leading to the recruitment of adapter proteins TRADD, which form death-inducing signaling complex (DISC) with caspase-8 and activates caspase-8 to induce downstream caspase-3 activation (Wang Z et al. 2005). C-FLIP can regulate and inhibit extrinsic pathway by competing with caspase-8 for DISC activation (Wilson NS et al. 2009). So upregulating pro-apoptotic TRAIL, DR5 and caspase-8 or downregulating anti-apoptotic FLIP may be the mechanisms to promote apoptosis through the extrinsic pathway.

PI3K-Akt pathway can be activated by a variety of extracellular signals, and involved in cell proliferation, survival and protein synthesis, and tumor growth. The activation of Akt can inhibit apoptosis through activating pro-survival factors like NF-κB and inactivating pro-apoptotic factors such as Bad (a Bcl-2 family member), FOXOs, caspase-9, as well as GSK3β (Jiang BH et al. 2008; Dillon RL et al. 2007). Moreover, Akt signaling has been found to be required in the functions of Wnt-1, 3a, 5a, and 8a in different systems (Longo KA et al. 2002; Almedida M et al. 2005; Constantinou T et al. 2008; Naito AT et al. 2005), suggesting the critical role of Akt signaling in Wnt cascade which is involved in cell survival.

GSK3β, which can inhibit Wnt/β-catenin signaling, has also been shown to be associated with cell apoptosis and inflammation. Overexpression of GSK-3β is sufficient to trigger apoptosis in different types of cells, such as neurons, vascular smooth muscle

cells, endothelial cells, and astrocytes (Li M et al. 2000; Hall JL et al. 2001; Kim HS et al. 2002; Sanchez JF et al. 2003). And GSK-3β has been found to promote the intrinsic apoptotic pathway triggered by different cellular insults, such as DNA damage, ER stress, hypoxia, removal of NGF or BDNF, hypertonic stress, oxidative stress, mitochondrial toxins and ceramide, by regulating transcription factors that control pro- and anti-apoptotic proteins, by promoting microtubule disruption, and by inducing mitochondrial disruption though activating pro-apoptotic members like Bax or degrading anti-apoptotic members like Mcl-1 (Beurer E et al. 2006; Forde JE et al. 2007). On the other hand, Lithium, the inhibitor of GSK-3β, reduces mouse acute renal failure induced by LPS via attenuating inflammation and renal cell apoptosis (Wang Y et al. 2009), further indicating that GSK-3β plays a positive role in promoting cell apoptosis and inflammation.

For GSK3β, direct phosphorylation of N-terminal Ser9 is associated with the inhibition of GSK3β activity (Forde JE et al. 2007), and the activation of Akt leads to the phosphorylation of Ser9, thereby inhibiting GSK3β (Pearl LH et al. 2002). So Akt activation through phosphorylation at Ser473 and GSK inactivation through phosphorylation at Ser9 may be negatively correlated with cell apoptosis.

Also, ROS can be another mechanism that induces apoptosis. ROS has been shown to induce mitochondrial permeability transition (Garrido C et al. 2006). Furthermore, it was found that the oxidation of cardiolipin, which is a mitochondria-specific anionic phospholipid, decreases the affinity of cytochrome c to cardiolipin and leads to the detachment of cytochrome c from the inner mitochondrial membrane, which may be another mechanism to explain the role of oxidative stress in cell apoptosis (Ott M et al. 2007). Also, peroxide contributes to the induction of apoptosis by stimulating the

activity of nuclear transcription factors to upregulate pro-apoptotic proteins or to inhibit anti-apoptotic proteins (Chandra J et al. 2000). Some studies showed that ROS-induced apoptosis is related to Akt signaling. Manumycin-A stimulated superoxide production and apoptosis in lymphoid tumor and myeloma cell lines, and led to dephosphorylation of Akt in ROS-dependent manner (Sears KT et al. 2008). And Cao J et al further revealed a direct interaction between Akt and ROS: ROS mediated a conformational change of Akt by forming an intramolecular disculfide bond and disrupted Akt activity by promoting dephosphorylation at Ser473 (Cao J et al. 2009).

Consequently, to study the possible mechanisms of the increased apoptosis by nuclear Wnt13 forms, here we have investigated the effect of Wnt13 forms on caspase activation and expression, Bcl-2 family members including Bax, Bcl-2 and Bim, apoptotic factors in the extrinsic pathway, the Akt-GSK signaling and ROS production.

5.3 Materials and methods

5.3.1 Materials

Geneticin (G-418) were purchased from Invitrogen. The rabbit polyclonal antibodies against cleaved caspase-7 (Asp198), and caspase-7, Bax, Bim, Akt, P-Akt (Ser473), GSK3β, P-GSK3β (Ser9) were products from Cell Signaling Technology. The rabbit polyclonal Bcl-2 antibody was from Santa Cruz. EnzChek® Caspase-3 Assay Kit was from Molecular Probes.

5.3.2 Cell culture and transfection

Cell culture and transient transfection were described in 3.2 and 3.3. For stable transfection, BAECs were plated into a 6-well plate and each well was transfected transiently with either PCR3 vector control or one of Wnt13 constructs using Exgene500. 40 hours later, cells in each well were trypsinized and plated into an individual dish, and maintained with culture media supplemented with 750μg/ml Geneticin for 2-3 weeks till colonies appeared. After selection, cells positive for plasmid were remained and amplified for the use of experiments.

5.3.3 Plasmid constructs

Please refer 3.4.

5.3.4 RNA isolation and real-time PCR (refer 3.5)

5.3.5 Cell extracts and western blot analysis (refer 3.6)

5.3.6 Caspase-3 like activity assay

Caspase-3 like activity was determined using EnzChek® Caspase-3 Assay Kit. According to the manufacture's handbook, cells were harvested after treatment by

scrapping off the plates and subsequently underwent centrifuge at 600g for 5 minutes. After washing with PBS, cell pellet of each sample was lysed by 100µl 1X cell lysis buffer supplemented with 10mM NaF, 1X Phosphatase Cocktail and Apotonin (Sigma), and underwent freeze-thaw cycle. The lysed cells were then centrifuged at 5000rpm for 5 minutes (4^{o}C). 25µl of supernatant was used for caspase-3 like activity assay, and another 20µl of supernatant was used to determine protein content.

For caspase-3 like activity assay, 25µl of supernatant sample was transferred to a 96 well microplate and mixed with 75µl substrate working solution, which was a mixture of 25µM Z-DEVD-R110 and 1X reaction buffer at room temperature. At the same time, to generate standard curve, 5mM R110 standard solution was diluted to yield R110 solutions ranging in concentration from 0- 25µM, which were then transferred to the same microplate. After 30 minutes, the fluorescence intensity was measured by Fusion Microplate Reader (excitation/emission 475/520nm). Once the standard curve was drawn, the fluorescence intensity of each sample was calculated, and divided by protein content of the same sample for correction.

5.3.7 Protein determination

Protein content was measured using Bio-Rad *DC* Protein Assay kit, based on Lowry assay. Firstly, 10µg/µl BSA was diluted into the cell lysis buffer used in caspase-3 activity assay, to make 5 different dilutions for protein standard. For each 20µl sample and protein standard, 100µl Reagent A', which was made from Reagen A (alkaline copper tartrate solution) mixed with Reagent S) was added to the tube and mixed with the

sample. Then, 1ml Reagent B (dilute Folin Reagent) was pipetted into the tube containing Reagent A and the sample/standard, and then mixed with by vortex. After 15 minute incubation at room temperature, the reaction solution of each sample was transferred into a 96 well microplate, and the absorbance was read at wavelength 750nm by spectrophotometer. When the standard curve was drawn, the protein content of each sample was calculated using the equation generated from the standard curve.

5.3.8 Determination of reactive oxygen species

5.3.8.1 CM-H_2DCFDA assay for intracellular ROS (peroxide, peroxyl radical, peroxynitrite, and nitric oxide)

5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) is a cell-permeable indicator and sensitive to intracellular ROS. The fluorescent signal used to detect the ROS results from the intracellular removal of the acetate groups of CM-H_2DCFDA and oxidation. BAECs were plated in a 24-well plate and then transfected with Wnt13-Flag constructs. After 40 hours, media were removed from the plate, and cells were washed with PBS once. Subsequently, cells were added with PBS (Ca^{2+}, Mg^{2+}) supplemented with CM-H_2DCFDA dye (final concentration 2μM) and incubated at 37^{o}C for 30 minutes. Then cells were washed with PBS (Ca^{2+}, Mg^{2+}) for 3 times followed by incubation at 37^{o}C for 15 minutes. The fluorescence intensity was read using Fusion Microplate Reader at wavelength 490 (Excitation) /520 (Emission) nm. CyQuant assay was then performed to determine the cell number. The relative CM-

H_2DCFDA intensity was calculated by CM-H_2DCFDA fluorescence intensity/CyQuant fluorescence intensity.

5.3.8.2 Dihydroethidium (DHE) assay for superoxide

BAECs were plated in a 24-well plate and then transfected with Wnt13-Flag constructs. After 40 hours, media were removed from the plate, and cells were washed with PBS once. Subsequently, cells were added with no-serum DMEM supplemented with DHE dye (final concentration 10 μM) which upon reaction with superoxide anions forms a red fluorescent product (ethidium), and incubated at 37^{o}C for 30 minutes. Cells were then washed with PBS (Ca^{2+}, Mg^{2+}) for 3 times. The fluorescence intensity was read using Fusion Microplate Reader at wavelength 520 (Excitation) /590 (Emission) nm. CyQuant assay was then performed to determine the cell number. The relative DHE intensity was calculated by DHE fluorescence intensity/CyQuant fluorescence intensity.

5.3.8.3 CyQuant assay

The CyQUANT assay is based on the use of the green-fluorescent CyQUANT GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. Also cellular DNA content is highly regulated, and it is closely proportional to cell number, so the CyQUANT assay is one of the methods to measure cell number. After the CM-H_2DCFDA or DHE assay, PBS was removed from the plate and cells were frozen in -80^{o}C. After the freeze-thaw cycle, the plate were read by Fusion Microplate Reader at wavelength 485 (Excitation) /520 (Emission) nm for determination of background fluorescence. Then cells were added with a mixture of 1× CyQuant substrate and 1× lysis buffer followed by 10-minute incubation at room temperature. Then the fluorescence

intensity was read using Fusion Microplate Reader at 485 (Excitation) /520 (Emission) nm. The CyQuant fluorescence intensity was calculated by (Intensity after adding substrate – Intensity before adding substrate).

5.3.9 Statistical analysis

All results are expressed as mean ± SEM. All values in this chapter are represented by the relative levels over PCR3 control. Since all the values in the control groups were set as 1, which does not fit normal distribution, One Way ANOVA analysis is not appropriate at this point. Thus, One Sample T-test (hypothesized value = 1) was used. Statistical significance was accepted at a value of $P<0.05$.

5.4 Results

5.4.1 The effect of Wnt13 isoforms on caspases in BAECs

5.4.1.1 Caspase-3 activation

Caspase-3 activation is one of the hallmarks during apoptosis, so we firstly determined caspase-3 like activity in BAECs. As shown in Figure 5.1A, after transient transfection with different Wnt13-Flag constructs, the caspase-3 like activity at basal levels was increased only by the nuclear M1L-Wnt13B (around 60%) compared to the vector control PCR3. After 100ng/μl LPS treatment for 16 hours, caspase-3 activity went up to 8 fold higher than that in basal level (data not shown), and M1L-Wnt13B showed increased activity by 50% compared to the vector control. Although this increase was not

statistically significant, the increase trend is still similar than that in basal levels. We also established BAECs which stably expressed Wnt13-Flag isoforms, and similar to transient transfection, M1L-Wnt13B still showed 1.5 fold increase of caspase-3 like activity compared to the vector control both at basal levels and after LPS treatment. But unlike transient transfection, stably-transfected Wnt13C (Figure 5.1B) had up to 2 fold increase of caspase-3 activity than control group in LPS-treated BAECs. For M74L-Wnt13B, there was a moderate increase (40%-50%) in transient transfection, but the increase in stable transfection was even bigger (by 80%), especially in basal levels showing the statistical significance.

To further confirm caspase-3 activation, we also measured the amount of protein product after caspase-3 cleavage by western blotting. Two markers were used: one is cleaved caspase-3/actin, which demonstrated the absolute amount of cleaved caspase-3 produced during apoptosis; the other is cleaved caspase-3/total caspase-3, which represented the ratio of cleavage compared to total caspase-3 (pro-caspase-3 plus cleaved-caspase-3). There was no cleaved-caspase 3 detected at basal levels, but LPS treatment induced obvious caspase-3 cleavage in BAECs. In transient transfection (Figure 5.2A), M1L-Wnt13B showed around 2 times increase in cleaved caspase-3/actin during LPS-induced apoptosis, and the mitochondrial forms (Wnt13B and M74L-Wnt13B) increased the amount of cleaved caspase-3 to a less extend (around 50%). Also, cleaved caspase-3/total caspase-3 ratio was increased by mitochondrial forms and nuclear forms, but all the Wnt13 isoforms showed less increase of cleaved caspase-3/total caspase-3 than that of cleaved caspase-3/actin, especially M1L-Wnt13B (40% increase in cleaved caspase-3/total caspase-3), suggesting that the increase of caspase-3 cleavage

may partially result from the increase of pro-caspase-3 expression. However, in stable transfection (Figure 5.3A), Wnt13C-Flag was the only form to obviously elevate cleaved caspase-3/actin (2.3 fold), and M1L-Wnt13B and M74L-Wnt13B had a moderate increase by 50%. But the ratio of cleaved caspase-3/total caspase-3 remained unchanged for all the Wnt13 isoforms, further suggesting the increase of pro-caspase 3 levels by Wnt13 forms.

5.4.1.2 Caspase-3 expression

We then analyzed caspase-3 expression at protein levels and mRNA levels. As expected, transiently-transfected M1L-Wnt13B had bigger increase (> 2 fold) of capase-3 protein expression both at basal levels and after LPS treatment than all the other Wnt13 forms, as is shown in Figure 5.4A, supporting the previous hypothesis that increased caspase-3 cleavage was partially due to the increase of pro-caspase-3 expression. At mRNA levels, M1L-Wnt13B still produced higher increase of caspase-3 than other Wnt13 isoforms (Figure 5.4B). However in stable transfection (Figure 5.5), all the Wnt13 isoforms increase both the protein and mRNA expression of caspase-3 at basal level more than Wnt13B, which can be explained by the stably-transfected Wnt13B expression level which was the least among all the isoforms. After LPS treatment, the protein level of caspase-3 went up to 4 fold of the expression at basal levels, and all the Wnt13 isoforms, showed a trend of increase (more than 50%), especially M1L-Wnt13B and M74L-Wnt13B (> 2 fold), but only Wnt13A had statistical significance.

5.4.1.3 Caspase-7 cleavage and expression

The EnzChek® Caspase-3 Assay Kit we used for caspase-3 like activity allowed the detection of not only caspase-3 activity, but also other DEVD-specific protease activities, such as caspase-7. Therefore, we also determine the cleavage of caspase-7 to see whether caspase-7 contributed to the increase of caspase-3 like activity. As shown in Figure 5.6A, Wnt13A, Wnt13B and M1L-Wnt13B increased the absolute amount of cleaved caspase-3 (cleaved caspase-3/actin) in transiently-transfected BAECs during LPS-induced apoptosis. However, the ratio of cleaved caspase-7/caspase-7 was not changed by any Wnt13 isoforms. Thus we then investigated the caspase-7 expression, and we found that Wnt13 forms, except Wnt13C and M74L-Wnt13B, showed a trend in increasing the protein expression of caspase-7 at basal levels. LPS treatment induced a 4 fold increase in protein levels of caspase-7 compared to basal levels (data not shown), and Wnt13A and M1L-Wnt13B further elevated caspase-7 protein expression (Figure 5.6B). Wnt13B showed a similar increase, but it was not statistically significant. Therefore, Wnt13A and M1L-Wnt13B increase the amount of cleaved caspase-7 mainly by increasing caspase-7 expression.

5.4.2 The effect of Wnt13 forms on Bcl-2 family members in BAEC

5.4.2.1 Bax/Bcl-2

Bcl-2 family governs the switch on or off of apoptosis by the pro-apoptotic members such as Bax and anti-apoptotic members such as Bcl-2. Therefore, to study the underlying mechanisms by which nuclear Wnt13 forms increase the caspase activation,

we analyze the levels of Bax and Bcl-2. Firstly, in transient transfection (Figure 5.7A), both protein levels and mRNA levels of Bax were shown upregulated (around 50%) by M1L-Wnt13B at basal levels in BAECs; during LPS-induced apoptosis, the protein levels of Bax were also increased by M1L-Wnt13B (Figure 5.7B). However, stably-transfected Wnt13 isoforms did not show any effect on Bax expression at either protein levels or gene levels (Figure 5.8). Moreover, in transiently-transfected BAECs at basal levels, M1L-Wnt13B and M74L-Wnt13B upregulated both the protein expression and mRNA expression of Bcl-2 by 80% and 60%, respectively, which is shown in Figure 5.7 and 5.9. After LPS treatment, the protein expression of Bcl-2 had a similar trend of increase by M1L-Wnt13B and M74L-Wnt13B (Figure 5.7B). Like Bax, the Bcl-2 expression in stable transfection was not obviously changed by Wnt13-Flag isoforms (Figure 5.8 and 5.10).

Importantly, Bcl-2 forms heterodimer with Bax to antagonize Bax/Bax homodimerization and disrupts Bax-induced apoptosis, so the ratio of Bax/Bcl-2 represents a cell-autonomous rheostat determining the life or death response of the cell to apoptotic stimuli (Korsmeyer SJ et al. 1995). We then calculated the Bax/Bcl-2 ratio which imbalance will cause the activation of downstream apoptotic events. Figure 5.11 demonstrated that for both transient transfection and stable transfection, the ratio of Bax/Bcl-2 remained unchanged by Wnt13 isoforms either at basal levels or after LPS treatment.

5.4.2.2 Bim expression

Besides Bax and Bcl-2, BH3 only proteins, such as Bim, represent one subfamily under Bcl-2 family, which function in activating Bax to promote pro-apoptotic action. Thus we determined the expression of Bim in BAECs. In transient trasfection (Figure 5.7 and 5.9), M1L-Wnt13B upregulated Bim expression at protein levels both at basal levels (>2 fold) and after LPS treatment (around 1.8 fold), and also upregulated its mRNA levels up to 4 fold at basal levels. Like M1L-Wnt13B, Wnt13B had a similar increasing effect but with lower extent. Wnt13A showed a trend of increasing Bim expression, but only the protein expression at basal levels had statistical significance. For stable transfection (Figure 5.8 and 5.10), like other markers described previously, Wnt13B induced lower Bim expression than all the other Wnt13-Flag isoforms (Figure 5.12). Nuclear forms of Wnt13 and M74L-Wnt13B increased Bim expression at mRNA levels by 2-3 fold. Moreover, only M74L-Wnt13B showed the statistical significance in increasing Bim expression at protein levels in LPS-treated BAECs. Therefore, although there was no difference in Bax/Bcl-2 ratio in Wnt13-transfected BAECs, the increase of pro-apoptotic Bim expression may be one of the mechanisms driving elevated caspase activation by nuclear Wnt13 forms.

5.4.3 The extrinsic pathway and inflammatory caspases

We were also wondering whether the increased caspase activation in BAECs was due to the change in apoptotic factors involved in extrinsic pathways, such as TRAIL, DR5, caspase-8 and anti-apoptotic FLIP. Realtime PCR analysis in transient transfection

showed that only M1L-Wnt13B had a trend in increasing mRNA levels of TRAIL and DR5, caspase-8 (Figure 5.12). Wnt13B demonstrated a weaker effect on increasing mRNA levels of caspase-8. However, the increasing trend in pro-apoptotic caspase-8 by M1L-Wnt13B and Wnt13B was accompanied by the same or even stronger increase in anti-apoptotic FLIP, suggesting that the extrinsic apoptotic pathway might not be the main force to drive EC apoptosis by Wnt13 forms.

Besides executioner caspases (caspase-3 and 7) and initiator caspases (caspase-8), we also tried to discover whether Wnt13 forms increase the expression of inflammatory caspases (caspase-4 and 5). As shown in Figure 5.13, Wnt13 forms did not show an increase in mRNA expression of caspase-4 and caspase-5 in transiently-transfected BAECs. Therefore, our findings showed that Wnt13 forms mainly increased the expression/activation of the executioner caspase-3/7, but not the extrinsic pathway factor caspase-8, nor inflammatory caspase-4/5.

5.4.4 Akt-GSK signaling and ROS production

Since PI3K-Akt is a pro-survival signaling, and the decrease in Akt activation is related to apoptosis, we thereby tried to investigate whether Wnt13 forms had effect on Akt signaling. The phosphorylation at Ser473 of Akt is required for the full activation of Akt, and we found out the P-Akt (Ser473)/Akt was not obviously changed by transient transfection of Wnt13 forms (Figure 5.14A). Moreover, Akt targets GSK3β, a kinase inhibiting Wnt/β-catenin signaling and promote apoptosis in its downstream activation events, so we determined the phosphorylation of GSK3β at Ser9 which is inhibitory to

GSK activity. As shown in Figure 5.14B, Wnt13 forms did not decrease this phosphorylation; instead, M1L-Wnt13B showed an increasing trend in GSK phosphorylation. Therefore, the increased apoptosis by Wnt13 forms is independent of Akt activation and GSK activation.

ROS can be one of the events that induce apoptosis with the involvement of Akt, so we then determined if Wnt13 forms affected ROS production in BAECs. As shown in Figure 5.15, all the Wnt13 forms were able to decrease the fluorescence intensity in CM-H_2DCFDA staining slightly (by 20%). However, Wnt13 forms did not decrease the fluorescence intensity in DHE staining; instead, mitochondrial forms increased the intensity of DHE by 20-40%, and Wnt13B was statistically significant. What these two dyes measure differs in species of ROS: the CM-H_2DCFDA probe is indicative of intracellular ROS, including peroxide, peroxyl radical, peroxynitrite, and nitric oxide whereas DHE dye is mainly indicative of superoxide. Thus, our results suggest that Wnt13 forms slightly decrease the amount of peroxide, peroxyl radical, peroxynitrite, and nitric oxide, while mitochondrial forms may have a mild increase in the production of ROS (mainly superoxide). Overall, Wnt13 forms showed limited effect on the production of total ROS, and the increased caspase-3 activation by Wnt13 fomrs may not be due to ROS production.

5.5 Discussion

Altogether the results presented in this study showed: 1) exogous nuclear Wnt13 forms increase caspase-3 like activity, cleavage of caspase-3,7 and caspase-3,7

expression in BAEC; 2) exogenous mitochondrial Wnt13 forms increased cleavage and protein expression of caspase-3,7, but not caspase-3 like activity; 3) except caspase-3 expression, secreted Wnt13A did not increase caspase-3 like activity and caspase-3 cleavage in BAECs; 4) Wnt13 forms were unable to change Bax/Bcl-2 ratio, but upregulated Bim expression; 5) Wnt13 forms did not significantly increase apoptotic factors (TRAIL, DR5, and caspase8) in the extrinsic apoptotic pathway; 6) Wnt13 forms did not increase inflammatory caspase-4 and caspase-5; 6) Wnt13 forms did not change the phosphorylation of Akt and GSK. Hence, Wnt13 forms increase apoptosis in BAEC with different strengths, and Wnt13 forms, especially nuclear forms, increase the activation of caspases possibly though upregulating the expression of caspases and Bim protein.

5.5.1 BAECs are sensitive to LPS-induced apoptosis

Bacterial lipopolysaccharide (LPS) is an outer envelope of gram-negative bacteria, and it has been shown to cause systemic inflammatory responses as well as the injury of BAECs in vitro (Chakravortty D et al. 2000). And endothelial cell death induced by LPS in a dose range is apoptotic in nature (Bannerman DD et al. 2003). In our study, BAECs started to undergo morphological changes, such as cell shrinking, rounding, and the formation of intercellular gaps after 100ng/ml LPS treatment for 6 hours. 16 hours later, LPS induced obvious caspase-3 and 7 cleavage (Figure 5.2 and 5.6), 8-9 fold of increase in caspase-3 like activity, and 4-5 fold of increase in the expression caspase-3

and caspase-7, suggesting that BAECs are the cell type sensitive to undergo apoptosis induced by LPS.

5.5.2 Wnt13 forms do not induce apoptosis at basal levels, but increase the susceptibility of BAECs to LPS-induced apoptosis

At basal levels, although the caspase-3 like activity and the expression of caspase-3, 7 were increased by Wnt13 forms, there was no caspase-3 cleavage detected in BAECs transfected with Wnt13-Flag constructs. However, LPS-challenged cells showed obvious cleavage of caspase-3, and Wnt13 isoforms, especially nuclear forms further increased caspase-3 cleavage, as well as caspase-3 like activity in BAECs treated with LPS, implying that Wnt13 proteins *per se* are not apoptotic inducers or stimuli, but the factors that promote the apoptotic process, in particular by increasing the expression of pro-apoptotic factors such as executioner caspase-3/7.

5.5.3 The nuclear forms of Wnt13 have stronger pro-apoptotic effect in BAECs than other forms of Wnt13

In our study, the nuclear forms of Wnt13 demonstrated robust effects on increasing apoptosis in BAECs due to the following facts: 1) the nuclear Wnt13 forms increased caspase-3 like activity (in both transient and stable transfection) with or without LPS treatment, caspase-3 cleavage (in transient transfection) and caspase-3 expression (in both transient and stable transfection) in BAECs (Figure 5.1-5.5); 2) the nuclear Wnt13 forms increased caspase-7 cleavage due to the increase of caspase-7 expression in BAEC treated with LPS (in transient transfection; Figure 5.6); 3) the nuclear Wnt13 forms upregulated expression of both Bax and Bcl-2 at both protein levels and mRNA levels in

transient transfection (Figure 5.7-5.10), without changing Bax/Bcl-2 ratio (Figure 5.11); 4) the expression of BH3-only protein Bim was upregulated by nuclear Wnt13 forms (transient transfection) both at basal levels and after LPS treatment (Figure 5.7), which may be one of the explanations for the increased caspase activation by nuclear Wnt13 proteins.

Mitochondrial forms of Wnt13 seem to have moderate effects on increasing apoptosis in BAECs because: 1) mitochondrial forms increased protein expression of caspase-3 (Figure 5.2-5.3), but not caspase-3 like activity in BAEC in transient transfection (Figure 5.1), so mitochondrial forms may be unable to reach the threshold of caspase expression to induce caspase activation; 2) M74L-Wnt13B increased caspase-3 expression at basal levels and caspase-3 like activity after LPS treatment in stable transfection (Figure 5.5); 3) transiently-transfected Wnt13B increased caspase-7 cleavage in BAEC treated with LPS (Figure 5.6). For the Bcl-2 family members, mitochondrial Wnt13 forms have weaker effects on increasing expression of Bax (around 50%, Figure 5.7-5.10) as well as Bcl-2 (Figure 5.7-5.10), and have no effect on Bax/Bcl-2 ratio (Figure 5.11). Besides, Wnt13B upregulated Bim expression up to 2 fold in transient transfection, and this increase was slightly lower than nuclear Wnt13 forms which upregulated Bim up to 2.7 fold (Figure 5.7 and 5.9).

Secreted Wnt13A form is the one with the least activity in increasing apoptosis in BAECs. Wnt13A upregulated caspase-3 expression (Figure 5.4 and 5.5), but Wnt13A was not able to increase caspase-3 like activity and caspase-3 cleavage in BAECs (Figure 5.1-5.3). And Wnt13A showed a mild effect on caspase-7 cleavage and caspase-7 expression (Figure 5.6). As for Bcl-2 family members, Wnt13A did not show the ability

of increasing in the expression of Bax or Bcl-2, nor Bax/Bcl-2 ratio (Figure 5.7-5.11), but Wnt13A displayed a moderate effect on upregulating Bim expression (Figure 5.7-10).

Therefore, the strength of Wnt13 isoforms in increasing BAEC apoptosis is: nuclear >mitochondrial >secreted forms. This phenomenon is consistent with the findings our laboratory has reported in 2006, *i.e.*, nuclear forms of Wnt13, other than mitochondrial forms or Wnt13A, increased the appearance of apoptotic nuclei as well as the sensitivity of BAECs to TNFα-induced apoptosis (Struewing IT et al. 2006). Furthermore, some other Wnts showed pro-apoptotic action in cells. For example, Wnt7b mediated macrophage-induced programmed cell death in the developing mouse eye (Lobov IB et al. 2005), and Wnt5a overexpression cause increased apoptosis in thymocytes *in vitro* (Liang H et al. 2007). Additionally, components of Wnt signaling including Fz receptors, GSK-3β, c-myc have been shown to function in increasing cell apoptosis (van Gijn ME et al. 2001; Li M et al. 2000; Askewe DS et al. 1991). So our study further indicates that together with other Wnts, Wnt13 proteins, especially nuclear Wnt13 forms, are important for the regulation of cell apoptosis.

5.5.4 The difference of expression patterns in transient transfection and stable transfection

In transient transfection, although Wnt13C and M1L-Wnt13B are equivalent in translated protein, the expression of Wnt13C is always much weaker than that of M1L-Wnt13B in normal culture condition (Figure 4.2A), so it is expected that M1L-Wnt13B showed bigger effect in increasing apoptotic markers in BAEC than Wnt13C.

However, stable transfection is another case. For Wnt13 expression, except Wnt13A, all the other exogenous Wnt13 forms could not be detected by immunoblotting using Flag antibodies. Based on the immnofluoresence staining, we found that the expression pattern is: Wnt13A>Wnt13C=M1L-Wnt13B>M74L-Wnt13B>Wnt13B (described in 3.10.2), which makes easier to understand some phenomenon in stable transfection: 1) the advantage of M1L-Wnt13B over other forms in increasing caspase-3 activation and expression was not that obvious (Figure 5.1-5.2 and Figure 5.4) due to the lower levels of M1L-Wnt13B in stable transfection (maybe more M1L-Wnt13 positive cells died during G418 selection because of the increased EC sensitivity by M1L-Wnt13B); 2) Wnt13C sometimes had stronger effects than in transient transfection (caspase 3 in Figure 5.1-5.3 and Bim in Figure 5.8, 5.10); 3) Wnt13A had higher incease in caspase-3 expression (Figure 5.5); 4) the effect of Wnt13B was the weakest in upregulating the expression of caspase-3 and Bim (Figure 5.5 and 5.8, 10). And we also noticed that Wnt13 forms in stable transfection had stronger effects than those in transient transfection. For example, the mRNA expression of caspase-3 was increased by Wnt13 forms up to 5 fold in stable transfection instead of 2 fold in transient transfection (Figure 5.4-5.5). A possible explanation is that in stable transfection, Wnt13 forms has been expressed continuously although in lower amount, which might produce durable and accumulated actions, ending up with a bigger effects on EC sensitivity to apoptosis.

5.5.5 The putative significance of Wnt13 forms in apoptosis

In the previous chapter (Aim1), our data demonstrated that nuclear Wnt13C was increased at translational levels during apoptosis induced by a variety of stress stimuli. And in this chapter, we have shown that nuclear forms of Wnt13 was able to increase

caspase activation through upregulating caspase and Bim expression, further suggesting the presence of the positive loop between expression and function of Wnt13 nuclear forms, which may facilitate the completion of apoptosis in BAECs upon stress challenge.

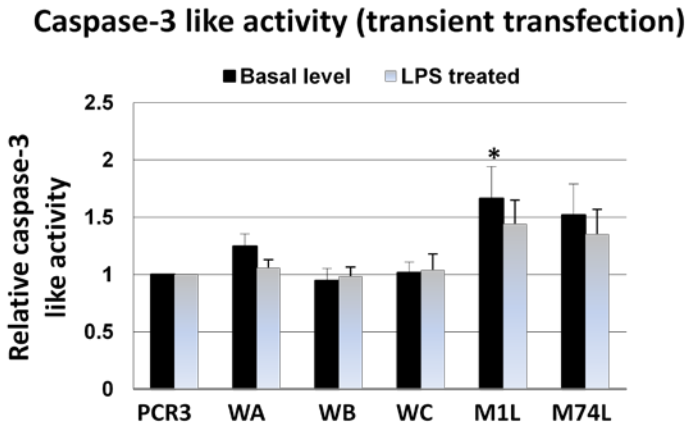
5.5.6 Wnt13, Akt/GSK signaling and ROS

PI3K-Akt pathway is involved in cell proliferation, survival and protein synthesis, and tumor growth through activating pro-survival factors like NF-κB and inactivating pro-apoptotic factors such as Bad, FOXOs, caspase-9, as well as GSK3β (Jiang BH et al. 2008; Dillon RL et al. 2007). Akt signaling is required in the functions of Wnt-1, 3a, 5a, and 8a in different systems (Longo KA et al. 2002; Almedida M et al. 2005; Constantinou T et al. 2008; Naito AT et al. 2005). GSK3β, which can inhibit Wnt/β-catenin signaling, has also been shown to promote cell apoptosis. The activation of Akt leads to the phosphorylation of GSK3β at Ser9, thereby inhibiting GSK3β (Pearl LH et al. 2002). So Akt activation through phosphorylation at Ser473 and GSK inactivation through phosphorylation at Ser9 may be negatively correlated with cell apoptosis. However, our study showed that Wnt13 forms did not significantly decrease either Akt phosphorylation at Ser473 or GSK3β phosphorylation at Ser9, indicating that Wnt13 forms did not inhibit Akt activation nor promote GSK activation in BAECs. Instead, M1L-Wnt13 showed a moderate increase in GSK (Ser9) phosphorylation, which suggests that the effect of M1L-Wnt13B on GSK3β is relatively independent of Akt activity, and other signaling pathway, such as ERK1/2 which also phosphorylates GSK3β at Ser9 (Kim SD et al. 2007) may be involved in the increased EC sensitivity to apoptosis by Wnt13 forms.

Also, increased ROS production can be another mechanism that induces apoptosis, and some studies showed that ROS-induced apoptosis is related to Akt signaling. In our study, CM-H_2DCFDA staining showed that Wnt13 forms slightly decreased the amount of ROS including peroxide, peroxyl radical, peroxynitrite, and nitric oxide, while DHE staining showed that mitochondrial forms had a mild increase in the production of ROS (mainly superoxide). The net effect of Wnt13 forms is limited on the production of total ROS. Thereby, the increasing effect on EC susceptibility to apoptosis by Wnt13 forms may be not attributed by increased ROS production, or by Akt-GSK signaling. In addition, for H2DCFDA staining, we used intensity of CyQuant staining for normalization. However, CyQuant staining is measuring cell number without indicating cell status, so it would be more appropriate to use oxidized DCFDA for the control of probe uptake and the activity of esterase in cells.

Altogether, our data showed that Wnt13 forms, especially the nuclear forms, increased EC sensitivity to LPS-induced apoptosis via upregulating the expression of key apoptotic factors such as the executioner caspases and the pro-apoptotic Bim, without affecting the extrinsic apoptotic pathway, inflammatory caspases, ROS production or Akt/GSK signaling. Thus, our next goal is to determine which transcription factor is activated or regulated by Wnt13 forms to increase the expression of executioner caspases and Bim.

A)



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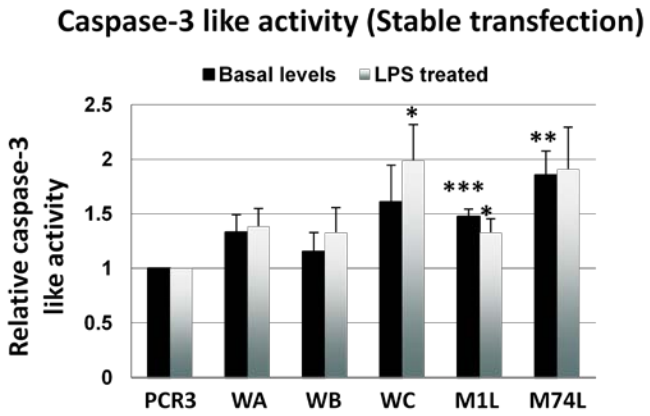
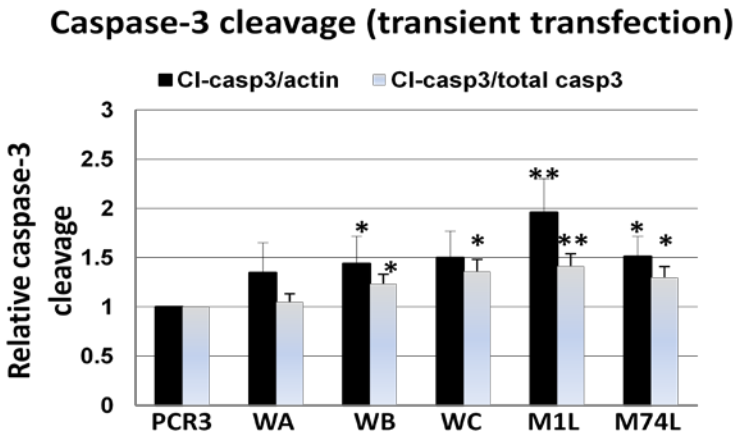


Figure 5.1 The nuclear Wnt13 forms increased caspase-3 like activity in BAECs. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 24 hours (A) or stably transfected (B), followed by treatment of 100ng/ml LPS; 16 hours later, cells were harvested and lysed for caspase-3 like activity determination. The relative activity levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6 independent transfection experiments; *<0.05, **<0.01, ***<0.001).

A)



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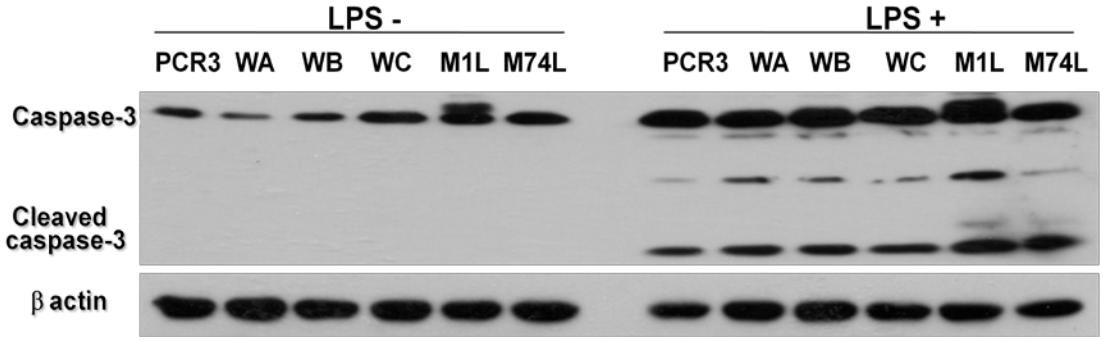
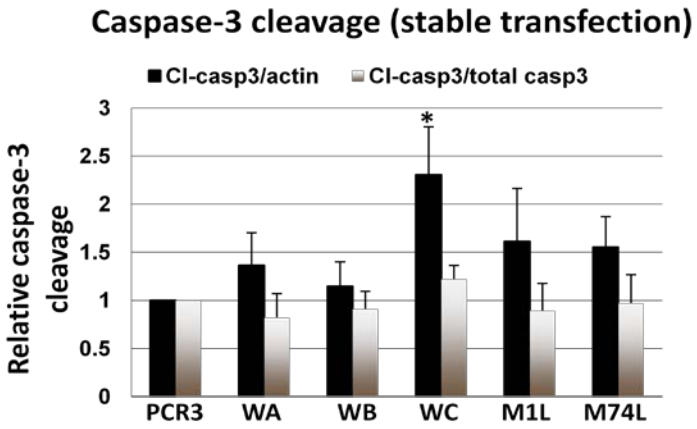


Figure 5.2 The nuclear and mitochondrial Wnt13 forms increased caspase-3 cleavage in BAECs in transient transfection. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 24 hours, followed by LPS treatment (100ng/ml). 16 hours later, whole cell exacts were prepared and Flag-tag, cleaved caspase-3 and β actin were analyzed by immunoblotting with specific antibodies. A) The relative cleavage levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6-15 independent transfection experiments; *<0.05, **<0.01). B) Representative images of western blotting analysis are shown.

A)



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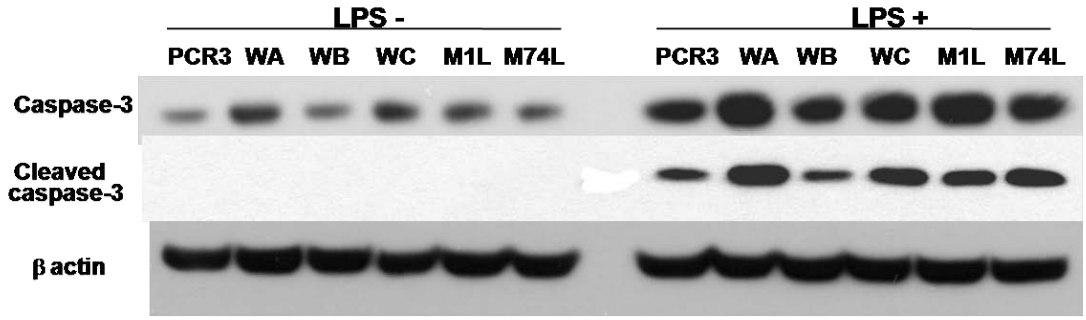
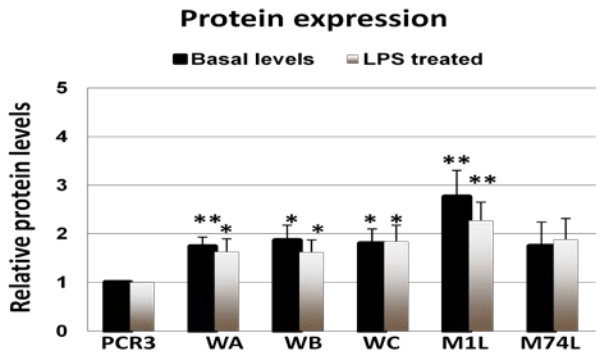


Figure 5.3 The nuclear Wnt13 forms increased caspase-3 cleavage in BAECs in stable transfection. BAECs were stably transfected with the PCR3 vector or Wnt13-Flag constructs, followed by LPS treatment (100ng/ml). 16 hours later, whole cell exacts were prepared and Flag-tag, cleaved caspase-3 and β actin were analyzed by immunoblotting with specific antibodies. A) The relative cleavage levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6-15 independent transfection experiments; *<0.05). B) Representative images of western blotting analysis are shown.

A)



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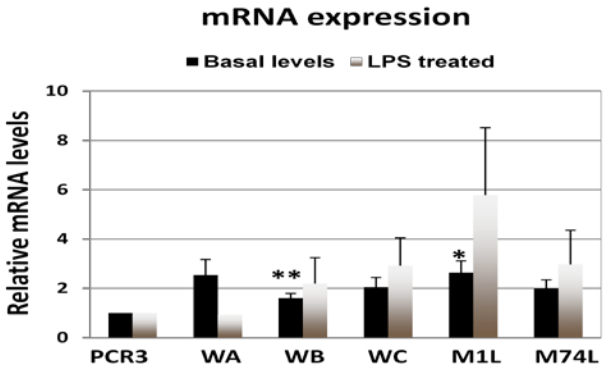
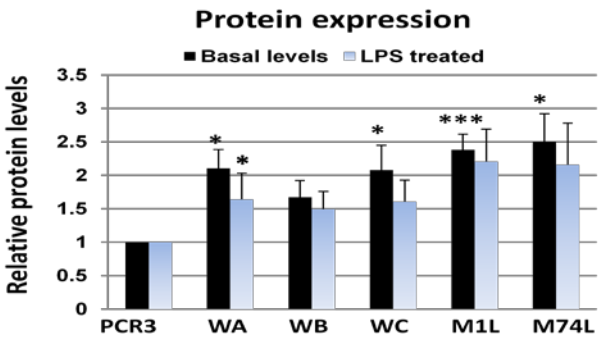


Figure 5.4 The nuclear Wnt13 forms increase caspase-3 expression more than the other forms in transient transfection. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 24 hours, followed by LPS treatment (100ng/ml), A) 16 hours later, whole cell exacts were prepared and caspase-3 and β actin were analyzed by immunoblotting with specific antibodies; or B) 6 hours later, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative protein or mRNA levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6-15 independent transfection experiments; *<0.05, **<0.01).

A)



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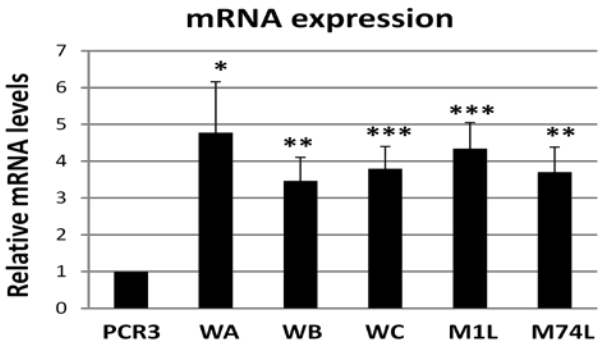
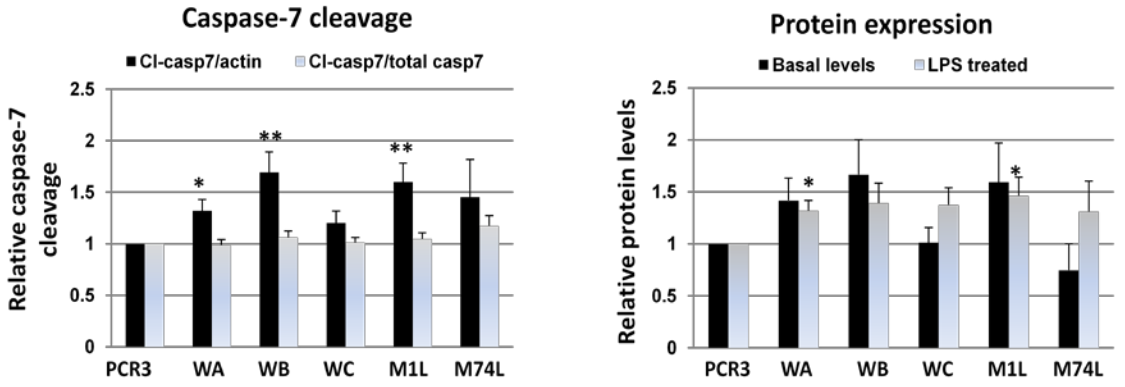


Figure 5.5 All the Wnt13 isoforms induced increase in caspase-3 expression at basal levels in stable transfection. BAECs were stably transfected with the PCR3 vector or Wnt13-Flag constructs, followed LPS treatment (100ng/ml). A) 16 hours later, whole cell exacts were prepared and caspase-3 and β actin were analyzed by immunoblotting with specific antibodies; or B) 6 hours later, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative protein or mRNA levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6 independent transfection experiments; *<0.05, **<0.01, ***<0.001).

A) B)



C)

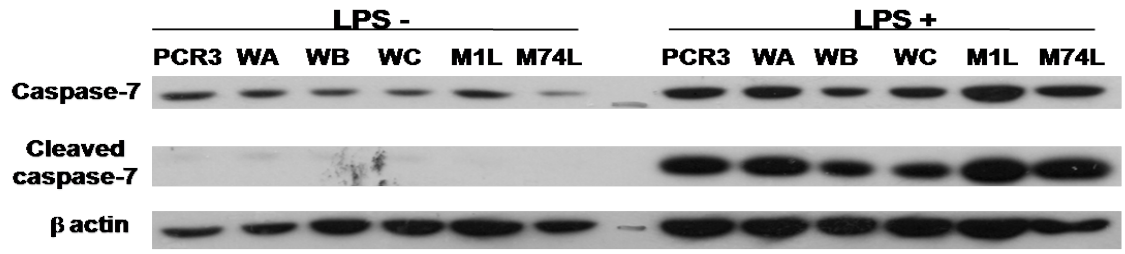
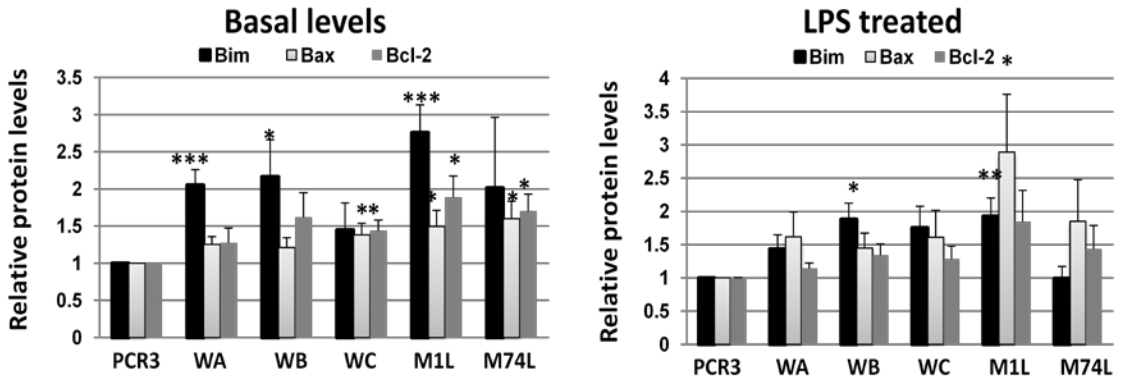


Figure 5.6 Transiently-transfected Wnt13 forms increase caspase-7 cleavage due to the increase of caspase-7 expression in BAECs treated with LPS. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 24 hours, followed by LPS treatment (100ng/ml). 16 hours later, whole cell exacts were prepared and cleaved caspase-7, caspase-7 and β actin were analyzed by immunoblotting with specific antibodies. The relative cleavage or protein levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=10 independent transfection experiments; *<0.05, **<0.01). Caspase-7 cleavage (A), the protein expression of caspase-7(B), and representative images of western blotting analysis (C) are shown.

A) B)



C)

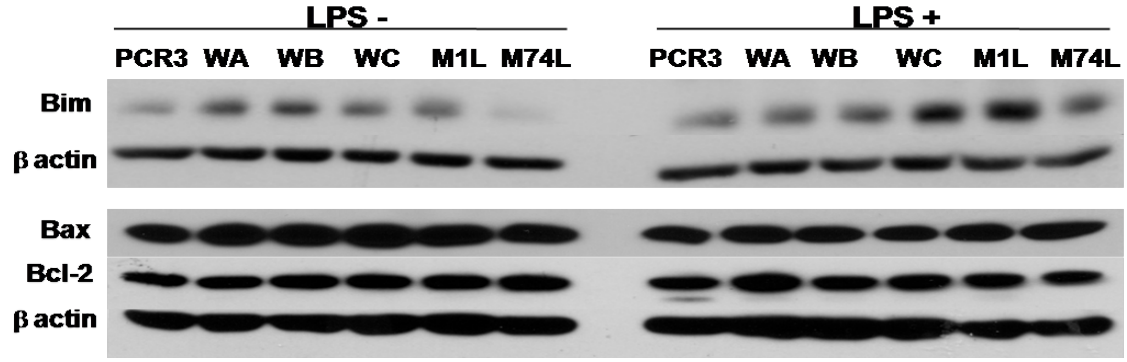
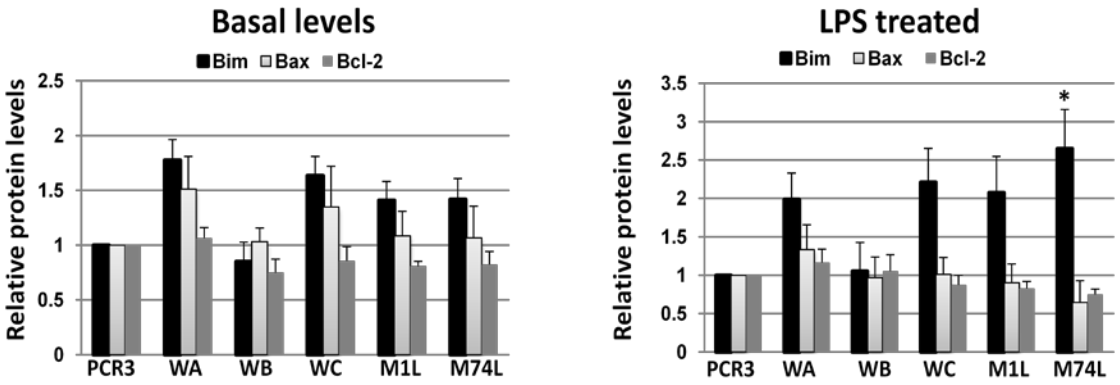


Figure 5.7 Effect of Wnt13 forms on the protein expression of Bcl-2 family members in transient transfection. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 24 hours, followed by LPS treatment (100ng/ml). 16 hours later, whole cell exacts were prepared, and Bim, Bax, Bcl-2 and β actin were analyzed by immunoblotting with specific antibodies. The relative protein levels after normalization with (A) basal or (B) LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=10 independent transfection experiments; *<0.05, **<0.01, ***<0.001). C) Representative images of western blotting analysis are shown.

A) B)



C)

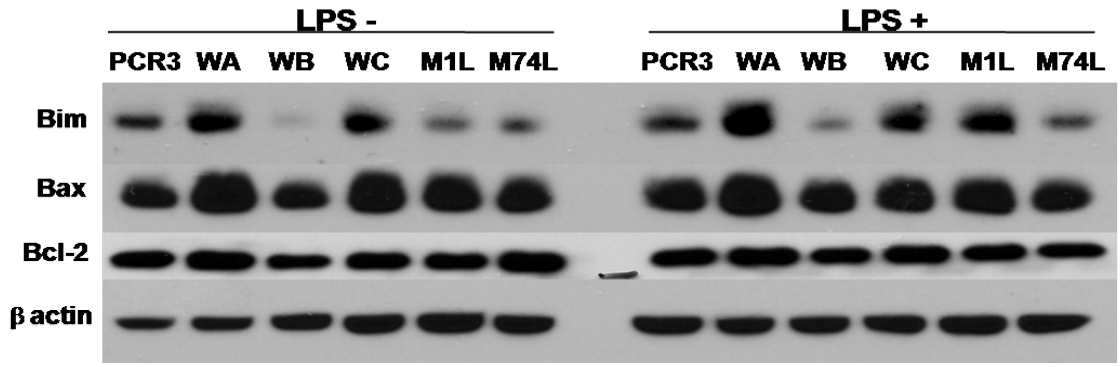
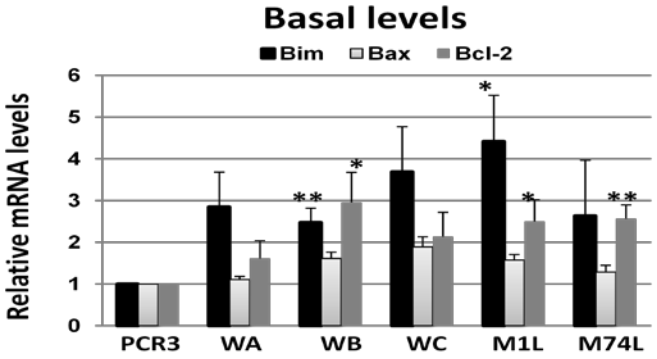


Figure 5.8 Effect of Wnt13 forms on protein expression of Bcl-2 family members in stable transfection. BAECs were stably transfected with the PCR3 vector or Wnt13-Flag construct, followed by LPS treatment (100ng/ml). 16 hours later, whole cell exacts were prepared and Bax and β actin were analyzed by immunoblotting with specific antibodies. The relative protein levels after normalization with (A) basal or (B) LPS-treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6 independent transfection experiments; *<0.05). C) Representative images of western blotting analysis are shown.

A)



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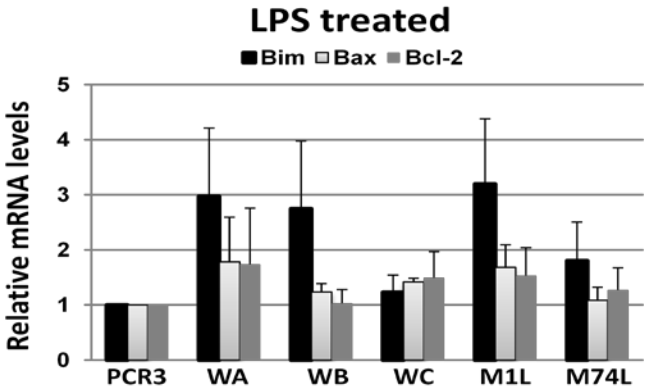


Figure 5.9 Effect of Wnt13 isoforms on mRNA expression of Bcl-2 family members in transient transfection. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 24 hours, followed by LPS treatment (100ng/ml). 6 hours later, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative mRNA levels after normalization with A) basal or B) LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=10 independent transfection experiments; *<0.05; **<0.01).

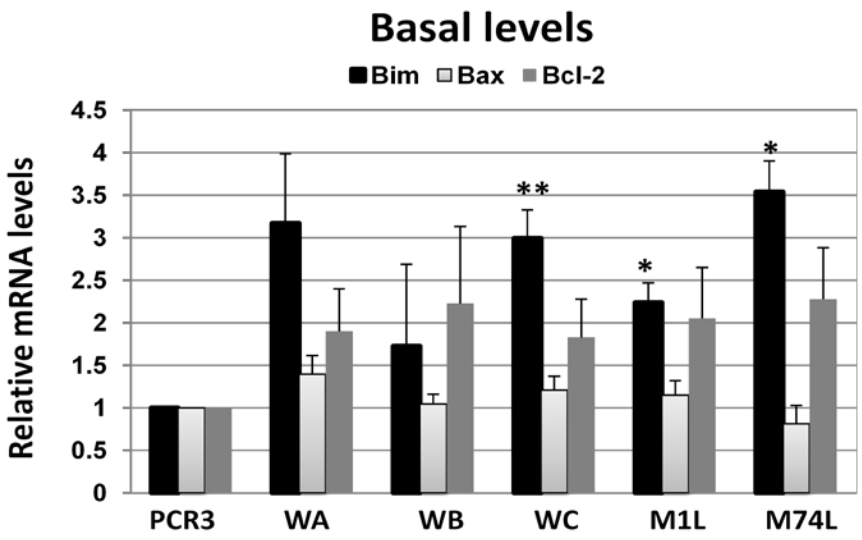
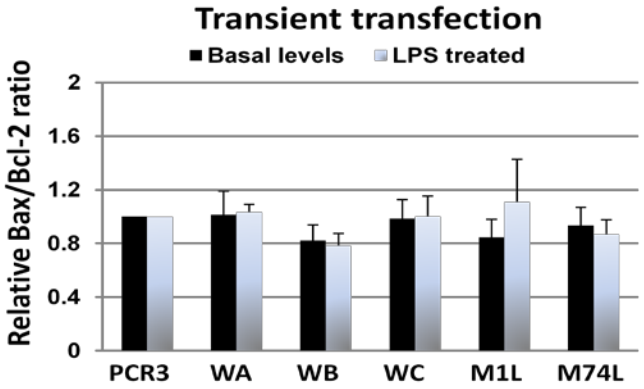


Figure 5.10 Effect of Wnt13 forms on mRNA expression of Bcl-2 family members at basal levels in stable transfection. BAECs were stably transfected with the PCR3 vector or Wnt13-Flag constructs. Cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative mRNA levels after normalization with basal PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6 independent transfection experiments; *<0.05; **<0.01).

A)



B)

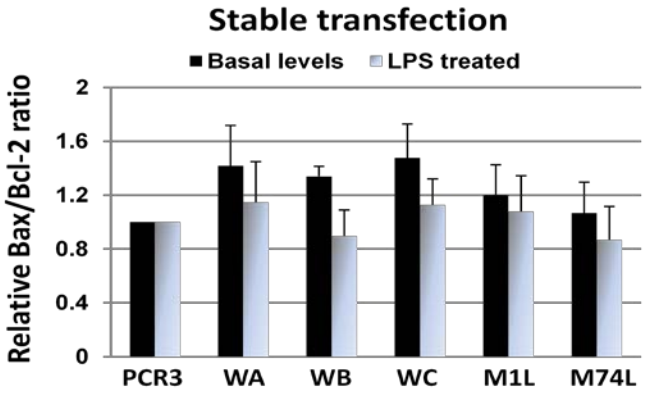
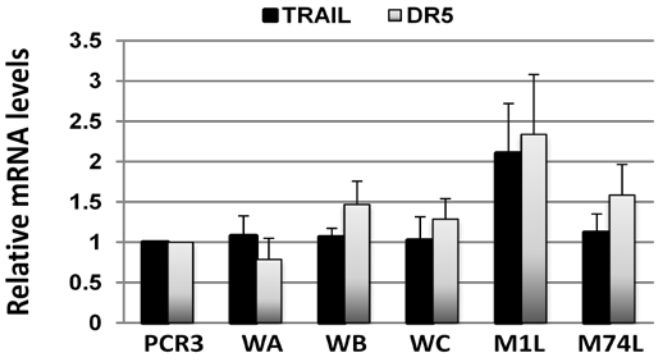


Figure 5.11 The Bax/Bcl-2 ratio remained unchanged by Wnt13 isoforms. BAECs were A) transiently transfected for 24 hours or B) stably transfected with the PCR3 vector or Wnt13-Flag constructs, followed by LPS treatment (100ng/ml). 16 hours later, whole cell exacts were prepared and Bax, Bcl-2, and β actin were analyzed by immunoblotting with specific antibodies. The relative ratio levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6-10 independent transfection experiments).

A)



B)

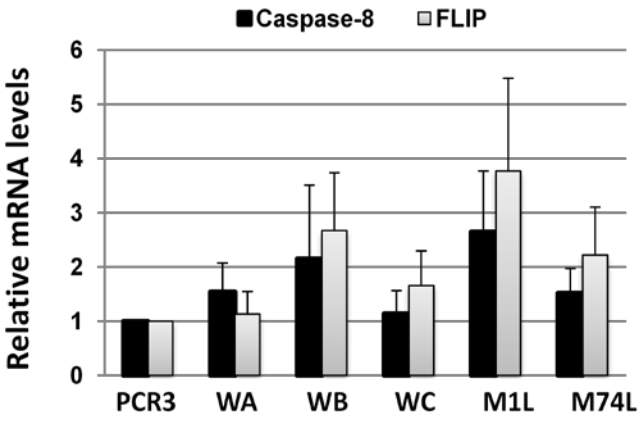


Figure 5.12 Effect of Wnt13 forms on the mRNA levels of extrinsic apoptotic factors in transiently-transfected BAECs. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 40 hours. Subsequently, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative mRNA levels after normalization with basal PCR3 values (set as 1) are represented in the graph (mean±SEM, n=5 independent transfection experiments.).

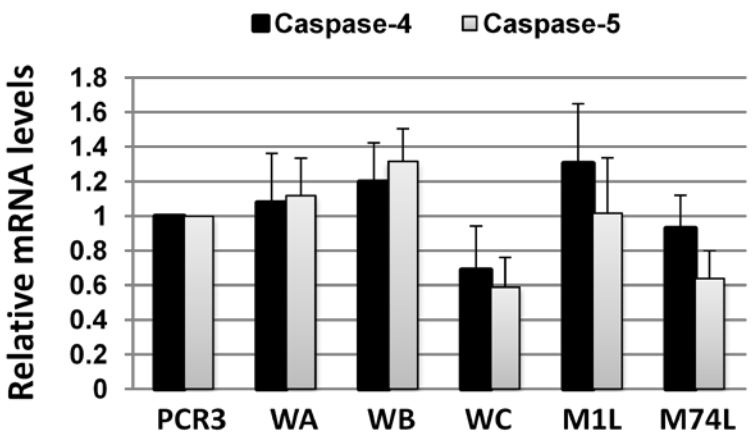
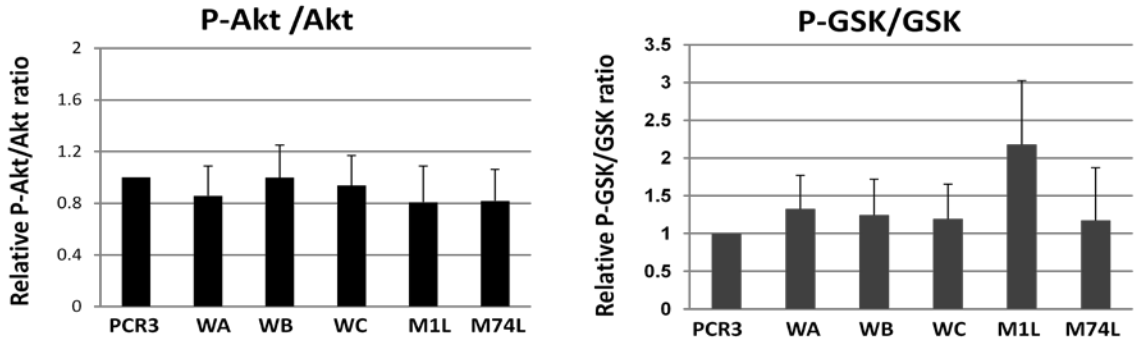


Figure 5.13 Effect of Wnt13 forms on the mRNA levels of inflammatory caspases in transiently-transfected BAECs. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 40 hours. Subsequently, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative mRNA levels after normalization with basal PCR3 values (set as 1) are represented in the graph (mean±SEM, n=5 independent transfection experiments.).

A) B)



C)

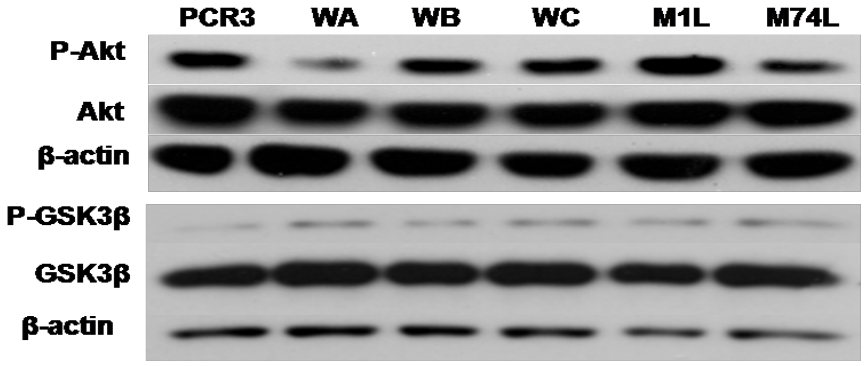


Figure 5.14 Effect of Wnt13 forms on phosphorylation of Akt and GSK3β. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 40 hours. Whole cell exacts were prepared, and A) Akt, and P-Akt (Ser473) or B) GSK3β and P-GSK3β (Ser9) were analyzed by immunoblotting with specific antibodies. The relative phosphorylation levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6-8 independent transfection experiments.).

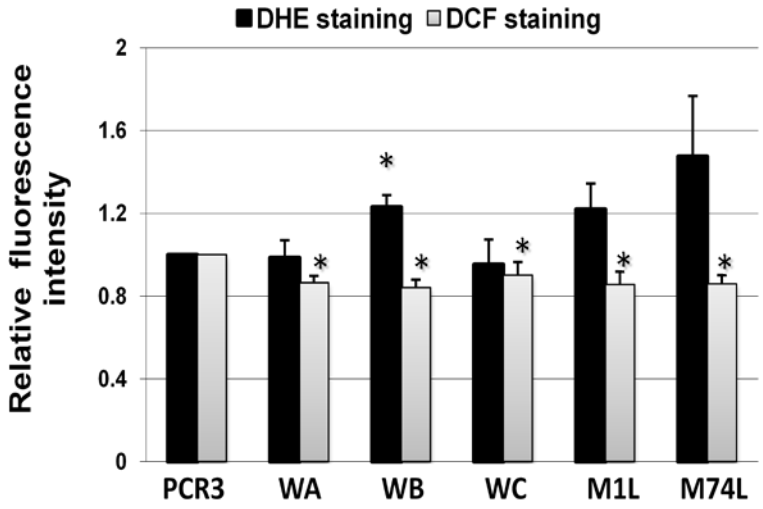


Figure 5.15 Effect of Wnt13 isoforms on the production of reactive oxygen species (ROS) in transiently-transfected BAECs. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag construct for 40 hours, followed by DHE staining and CM-H_2DCFDA (DCF) staining. The relative fluorescence intensity levels after normalization with basal PCR3 values (set as 1) are represented in the graph (mean±SEM, n=8-20 independent transfection experiments; *<0.05).

CHAPTER 6. SPECIFIC AIM 3

To study whether Wnt13 forms have differential effects on the expression and activation of FOXOs that mediate the transcriptional regulation of FOXO target genes related to apoptosis and oxidative stress resistance.

6.1 Summary

Wnt proteins control cell fate during development and postnatal homeostasis by regulating cell proliferation, cell differentiation and cell apoptosis. In the previous chapter, we have shown that Wnt13 forms increase apoptosis in BAEC with different strengths: nuclear > mitochondrial> secreted forms, and Wnt13 forms increase the activation and expression of caspases possibly though upregulating Bim protein. Therefore, it is interesting to know what upstream factors mediate the effect of Wnt13 forms on Bim and caspases as well as subsequent apoptosis. In this study, we found that in transient transfection, 1) nuclear Wnt13 forms increased the expression FOXO3a but not FOXO1; 2) Wnt13A and mitochondrial Wnt13 forms reduced the phosphorylation of FOXO3a at Ser253 which is a Akt site, but not FOXO1; 3) Wnt13A increased the nuclear localization of exogenous FOXO1 and endogenous FOXO3a, and M1L-Wnt13B also increased the nuclear localization of exogenous FOXO1 and FOXO3a; 4) M1L-Wnt13B upregulated the expression of a subset of FOXO target genes such as *SOD2*; 5) Wnt13 forms did not have significant effect in luciferase activity of Forkhead responsive element (FHRE from *FasL* promoter) reporter system and *SOD2* promoter; 6) however, M1L-Wnt13B increased the luciferase activity of *SOD2* intron 2 element (I2E) upon LPS treatment; 7) also, a putative FOXO site was found in intron 2, which was responsive to

activated FOXO3a form. Therefore, our conclusions are that Wnt13 forms increase FOXO localization in nucleus through either upregulating FOXO3a expression (M1L-Wnt13B) or decreasing FOXO3a phosphorylation (Wnt13A) and thereby upregulate the expression of a subset of FOXO target genes related to oxidative stress resistance; although it is not clear whether Wnt13 forms increase FOXO activity at Bim promoter, M1L-Wnt13B increased *SOD2* transcription upon LPS treatment possibly through a putative FOXO site at intron 2 in *SOD2* gene.

6.2 Introduction

Wnt signaling is recognized as one of the handful powerful pathways that control basic development and organism homeostasis (Clevers H. 2006 and Staal FJ et al. 2008). Wnts and Wnt signaling function in controlling cell fate, including cell proliferation or self-renewal, differentiation, senescence and apoptosis (Hayward P et al. 2008; Almeida M et al. 2005).

Wnt13, also named Wnt2b, is one of the rare members in Wnt family, which is expressed in a dynamic pattern during embryogenesis and organogenesis, and the expression is related to human diseases, including cancer, diabetes. The complexity of human Wnt13 gene has been shown in human cells with three isoforms identified: the secreted Wnt13A, mitochondrial Wnt13B and nucleus Wnt13C, which differ in their N-terminal sequences and generated through alternative promoter, alternative splicing and alternative start codons (Struewing IT et al. 2006; Bunaciu RP et al. 2008). Moreover, Wnt13 nuclear forms showed an increased sensitivity to LPS or TNF induced apoptosis

in primary endothelial cells (Struewing IT et al. 2006), and nuclear Wnt13C underwent translational regulation during apoptosis (Tang T et al. 2008). In the previous chapter (Aim 2), we have shown that Wnt13 forms increased EC sensitivity to LPS-induced apoptosis with different strengths: nuclear (robust) > mitochondrial (moderate) > secreted forms (weak), and Wnt13 forms increase the activation of caspases through upregulating the expression of caspases and pro-apoptotic Bcl-2 family member Bim which tips the balance of pro-apoptotic/anti-apoptotic factors in Bcl-2 family to pro-apoptosis. In this case, to discover the upstream factor, we started with FOXOs which are the common transcription factors that regulate both caspase-3 and Bim.

FOXO proteins are a family of transcription factors with important roles in metabolism, cell cycle, stress tolerance and possibly lifespan (van der Horst A et al. 2007). FOXO family consists of FOXO1, 3a, 4 and 6, and in differentiated endothelial cells, FOXO1 and 3a are the main forms of FOXO (Potente M et al. 2005). FOXO1-deficient mice died at around embryonic day 11 due to branchial arch defects and abnormal vascular remodeling in the yolk sacs, indicating that FOXO1 is required for mouse vascular development (Furuyama T et al. 2004). FOXO proteins undergo post-translational modifications like phophorylation, ubiquitination, acetylation and methylation, which either activate or inhibit the transcription activity of FOXOs (Huang H et al. 2007; Yamagata K et al. 2008). In the presence of growth factors, FOXOs located in the nucleus are phosphorylated by upstream kinases such as Akt and SGK on three conserved residues (Thr24, Ser256, and Ser319 for FOXO1; Thr32, Ser253, and Ser315 for FOXO3a). Phosphorylated FOXOs bind 14-3-3 protein, resulting in the export out of nucleus and subsequent degradation by proteasome in cytoplasm (Greer EL et al. 2005).

However, activated FOXOs (like FOXO1-AAA or FOXO3a-TM, in which 3 phosphorylation sites are mutated) stay in nucleus, and bind to the DNA binding element (DBE) of the target genes, initiating gene transcription. FOXO target genes include DNA repair-related *Gadd45*, oxidative detoxification-related *SOD2* and *catalase*, cell cycle-related $p27^{KIP1}$, *cyclin D1* and *cylin D2* (Huang H et al. 2007). In addition, FOXOs also target apoptosis-related genes such as caspase-3, *Bim*, as well as extrinsic pathway factors *FasL* and *TRAIL* (Bois PR et al. 2005; Huang H et al. 2007). FOXO1, 3, 4 triple-knockout mice developed thymic lymphomas and hemangiomas (endothelial cell tumor), suggesting that FOXOs are tumor suppressors and specifically important for endothelial cell homeostasis (Paik JH et al. 2007). The tumor suppressing effect of FOXOs may be related to their pro-apoptotic functions. Also, constitutively activated form FOXO1-TM has been shown to induce apoptosis in leukemia-derived cell lines via *TRAIL* (Kikuchi S et al. 2007); knock-down of FOXO3a by small interfering RNA abolished free fatty acid-induced hepatocyte apoptosis and Bim induction (Barreyro FJ et al. 2007), further indicating the promoting role of FOXOs in cell apoptosis.

In addition, FOXOs also converge with Wnt-signaling; in 2005, Essers MA discovered a functional interaction between FOXO and β-catenin under oxidative stress, so a new FOXO-Wnt model was then proposed: upon Wnt signaling which turns TCF on, or upon insulin signaling that turns FOXOs off, β-catenin prefers interacting with TCF rather than FOXO to promote cell proliferation; in contrast, under oxidative stress conditions where FOXO activity is on, β-catenin preferentially binds to FOXOs to induce apoptosis or quiescence (Essers MA et al. 2005; Bowerman B. 2005).

The status of oxidative stress can regulate cell apoptosis because oxidative stress is one of the inducers of intrinsic apoptotic pathway by promoting the permeability of mitochondria (Garrido C et al. 2006). *SOD2* and *catalase* are one of the subsets of FOXO target genes which belong to endogenous enzyme systems to defend oxidative stress. Superoxide (O_2^-) can be converted by SOD to H_2O_2, which is in turn converted to H_2O and O_2 by catalase; in mitochondria, MnSOD (the protein product of *SOD2*), is specifically used to catalyze the superoxide conversion (Kamata H et al. 1999). In SOD2 gene, the P7 fragment (-210 - +24) containing multiple Sp1- and AP-2 binding site, has a basal promoter function (Yeh CC et al. 1998). And two DBE sites for FOXO3a at promoter region were reported: one at -997 bp and the other one at -1, 249 bp, and only the latter one is critical for FOXO3a activity (Kops GJ et al. 2002). Also, several NF-κB sites in 5'- or 3'- flanking area were found to be irresponsive to the induction by TNF; however, the NF-κB site located in intron 2 element (I2E) is critical for TNF/IL-1-mediated induction (Xu Y et al. 1999), revealing the complexity of SOD2 regulation at transcriptional levels.

To test the hypothesis that FOXOs are involved in increased caspases and Bim by Wnt13 forms, we studied whether Wnt13 forms affect the expression, phosphorylation, nuclear localization and other target gene expression of FOXOs, as well as FOXO transcription activity by using Forkhead responsive element (FHRE) of *FasL* gene, and the transcriptional regulation of different SOD2 regions (promoter or intron regions).

6.3 Material and Methods

6.3.1 Materials

The rabbit polyclonal anti-phospho-FOXO1 (Thr24)/FOXO3a (Thr32), phospho-FOXO3a (Ser253), anti-FOXO1, anti-$P27^{kip}$ and anti-Creb as well as anti-COXIV antibodies were from Cell Signaling Technology. The rabbit polyclonal anti-MnSOD antibody was from Stressgene. The rabbit polyclonal anti-FOXO3a and the goat polyclonal anti-calnexin antibodies were purchased from Santa Cruz. The nuclear Extraction Kit was from Panomics. 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), Dihydroethidium (DHE), and th CyQuant® Cell Proliferation Assay kit were from Molecular Probes. The Dual Luciferase Reporter Assay System was from Promega and Galacto Light Plus was purchased from Tropix-Applied Biosystem.

6.3.2 Cell culture and transfection

Cell culture and transient transfection were described in 3.2 and 3.3. For stable transfection, please refer 5.3.2.

6.3.3 Plasmid constructs

Please refer 3.4 for Wnt13-Flag constructs. The plamids of pEGFP-N1-GFP-FOXO1, pEGFP-N1-GFP-FOXO1-AAA, pECE-HA-FOXO3a WT, pECE-HA-FOXO3a-TM, pECE-HA-FOXO3a-WT DBM (H212R) and FHRE-luc were purchased from

Addgene, which were then recovered, grown and amplified by maxipreps in our laboratory. PGL3-SOD2-P7-luciferase, PGL3-SOD2-1.6K-luciferase, PGL3-SOD2-3K-luciferase, and PGL3-SOD2-P7-I2E-luciferase constructs were kind gifts from Dr. Daret St Clair (University of Kentucky).

6.3.4 RNA isolation and real-time PCR (refer 3.5)

6.3.5 Cell extracts and western blot analysis (refer 3.6)

6.3.6 Immunofluorescence microscopy (refer 3.8)

6.3.7 Cell fractionation and nuclear extraction

The protocol for cell fractionation and nuclear extraction was based on manufactures' handbook and improved by our laboratory. Generally, for each sample, 10^6 BAECs were used and transfected with Wnt13-Flag constructs. After 40 hours, media were removed from the dish and cells were washed with PBS twice before 500µl hypotonic Buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA. 1mM DTT, 0.5 mM PMSF, 5 ul of protease inhibitor cocktail to 5 ml of buffer were added just before use) was added. Then the dish was placed on a rocker at 4^oC for 10 minutes. After scrapped off from the dish, cells were transferred to a 1.5ml microcentrifuge tube, and

syringed 20 times to break the cells. 50µl of cell suspension was taken out and saved as whole cell extract. The rest of 450µl suspension was centrifuged at 14,000g for 3 minutes (4^oC), and the supernatant was saved as the cytosol and the membrane. The pellet was then washed with Buffer A, and after the same centrifugation, the pellet was mixed with 150µl high salt Buffer B (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1M KCl; 10% Glycerol; 1mM DTT, 0.5 mM PMSF, 5 ul of protease inhibitor cocktail to 5 ml of buffer were added just before use), followed by nuclear extraction on a shaker for 1 hour (4^oC). After centrifuge at 14,000g for 10 minutes (4^oC), the supernatant was isolated and stored as nuclear extracts while the pellet was stored as unextracted nuclear fraction.

6.3.8 Dual luciferase reporter assay

According to the manufacture's instructions, each well (10^5 cells) of BAECs were co-transfected with 5 ng of pCMV-β-galactosidase construct for normalization purposes, and with 5 ng phRGTK (expressing Renilla luciferase to normalize for transfection efficiency), 250 ng PGL3 vector or luciferase constructs, and with 0.4 µg Wnt13-Flag constructs as well as 0.4 µg PCR3 vector. After 40 hours, cells were lysed with 125 µl Passive Lysis Buffer supplemented with proteasome inhibitors, and scrapped off the plate. Cells were then transferred to a microcentrifuge tube and centrifuged at 25,000 g for 5 minutes (4^oC). 20 µl of the supernatant was loaded into a 96-well microplate for luciferase assay with luciferase substrate for firefly activity and then Stop-Glo substrate for Renilla activity. Another 20 µl of the supernatant was loaded to another microplate for β-galactosidase activity assay, using Galacto Light Plus substrate for 30-minute

incubation prior to being added with accelerating reaction buffer. Both Renilla-firefly dual luciferase assay and β-galactosidase activity assay were performed using an LmaxII luminometer (Molecular Device). Although two internal controls (Renilla and β-galactosidase) were used, the corrected reporter luciferase activity was calculated by firefly luciferase activity/ β-galactosidase activity due to more consistency in β-galactosidase assay. All the transfection experiments were performed in duplicates.

6.3.9 Statistical analysis

All results are expressed as mean ± SEM. For the values represented by the relative levels over PCR3 control which was set as 1, One Sample T-test (hypothesized value = 1) was used when compared to PCR3 control. Statistical significance was accepted at a value of $P<0.05$. Otherwise, when the values of the control group fit normal distribution, One Way ANOVA analysis was used followed by Tukey's test for comparison between different groups.

6.4 Results

6.4.1 Effect of Wnt13 forms on FOXO expression in BAECs

Since increased FOXO expression may cause increased FOXO activity, we firstly investigated whether Wnt13 isoforms have an effect on the expression of FOXOs. In the experiments of transient transfection, we found that the expression of FOXO1 remained unchanged by Wnt13 forms (Figure 6.1A) at both protein levels and mRNA levels;

however, M1L-Wnt13B increased the protein expression of FOXO3a significantly, and all the intracellular isoforms upregulated the mRNA expression of FOXO3a by 2-2.5 fold (Figure 6.1B). In the experiments of stable transfection, Wnt13 forms did not show the significance in increasing FOXO1 expression either at protein levels or at mRNA levels (Figure 6.2A). But Wnt13 forms demonstrated a trend in increasing mRNA levels of FOXO3a, and M74L-Wnt13B was statistically significant (Figure 6.2B). And both graphs in Figure 6.2 showed that Wnt13B had less effect on the expression of FOXOs than all the other forms did, which can be explained by the expression pattern of Wnt13B which is the least among all the Wnt13 forms in stably-transfected BAECs as determined by imunofluoresence micoscopy.

6.4.2 Effect of Wnt13 forms on FOXO phosphorylation in BAEC

Post-translational modifications, such as phosphorylation, are regulatory events for FOXO activity. When FOXOs are phosphorylated by upstream kinases such as Akt and SGK on three conserved residues (Thr24, Ser256, and Ser319 for FOXO1; Thr32, Ser253, and Ser315 for FOXO3a), they bind 14-3-3 protein, resulting in the export out of nucleus and subsequent degradation by proteasome in cytoplasm (Greer EL et al. 2005). We used anti-phospho-FOXO1 (Thr24)/FOXO3a (Thr32) antibody which detects mainly FOXO1 phosphorylation because the expression of FOXO1 is 10 times more abundant than that of FOXO3a in BAECs (data not shown), and FOXO1 phosphorylation was shown unchanged by Wnt13 isoforms in transient transfection (Figure 6.3A). We also used specific anti-FOXO3a phosphorylation (Ser253) antibody and as shown in Figure

6.3A, Wnt13A and mitochondrial forms decreased FOXO3a phosphorylation at Ser253 by 40-60%, and nuclear forms of Wnt13 showed a moderate reduction in FOXO3a phosphorylation by 30-40% which was not statistically significant. As FOXO phosphorylation at Ser253 is inhibitory for FOXO transcriptional activity, the decreased FOXO3a phosphorylation by Wnt13 forms suggests that Wnt13 forms possibly increase FOXO activity.

6.4.3 Effect of Wnt13 forms on subcellular localization of FOXOs in BAEC

6.4.3.1 Exogenous localization of FOXOs

Decreased phosphorylation of FOXO3a may lead to less nuclear exclusion, so we then investigated the effect of Wnt13 forms on the subcellular localization of FOXOs in transient transfection. On one hand, we co-transfected GFP-FOXO1 or HA-FOXO3a with Wnt13-Flag into BAECs and detected the exogenous Wnt13-Flag forms and FOXOs by immunofluoresence microscopy. For PCR3 control (Figure 6.4), 50% exogenous FOXO1 was localized in the nucleus while 30% was localized in the cytosol. Wnt13A, Wnt13C, and M1L-Wnt13B were able to increase its nuclear localization up to more than 60% and decrease its cytoplasmic localization under 10%. However, as shown in Figure 6.5, PCR3 showed more exogenous FOXO3a in cytosol (45%) than in nucleus (40%). M1L-Wnt13B increased FOXO3a nuclear localization up to 80% and decreased its cytoplasmic localization down to 10%, and other Wnt13 forms such as Wnt13A and Wnt13B showed a trend in increasing the nuclear localization of FOXO3a, which was not statistically significant.

6.4.3.2 Endogenous localization of FOXOs

On the other hand, we isolated different subcellular fractions of BAECs and extracted the nucleus to see whether Wnt13 forms had similar effects on endogenous FOXOs. First, we used different markers to show the quality of subcellular fractionation and very little contamination among cell fractions (Figure 6.6C): COXIV, a mitochondrial protein, was only found in the fraction of cytoplasm plus membrane; both β-catenin and calnexin were mainly in the fraction of cytoplasm plus membrane, with a small amount in nucleus; and Creb is a nuclear transcription factor, which was localized both in extracted nucleus and in unextracted nucleus (around 1:1).

To our surprise, our data (Figure 6.6C) demonstrated that FOXO1 was totally absent in cytoplasm or membrane, but exclusively in the nuclear fraction (both in nuclear extracts and in unextracted pellet; around 1:6 in proportion). And Wnt13A or M1L-Wnt13B did not show obvious effect on FOXO1 localization in either nuclear extracts or in unextrated pellet (Figure 6.6A). However, unlike endogenous FOXO1, endogenous FOXO3a was shown localized both in the nucleus and in the fraction of cytoplasm plus membrane (Figure 6.6C). Wnt13A increased FOXO3a localization in nuclear extracts by more than 2 fold ($p=0.05$), and M1L-Wnt13B had a weaker increase (by around 50%). Wnt13A and M1L-Wnt13B showed mild decreasing effect on FOXO3a localization in the fraction of cytoplasm plus membrane. These results suggest that Wnt13A increased the nuclear localization of endogenous FOXO3a.

6.4.4 Effect of Wnt13 forms on the expression of FOXO target genes in BAEC

6.4.4.1 *$p27^{kip}$*

Since the expression of Bim and caspase-3 were shown upregulated by nuclear Wnt13 forms, we thereby tested whether Wnt13 isoforms increase the expression of other FOXO target genes, such as cell cycle-related *$p27^{kip}$*. As illustrated in Figure 6.7, transiently-transfected Wnt13A ($p<0.05$) and M1L-Wnt13B were able to upregulate the protein expression of *$p27^{kip}$* at basal levels by around 50%, and M1L-Wnt13B increased its mRNA expression by 5 fold. In stable transfection (Figure 6.8), Wnt13A ($p<0.01$) and M1L-Wnt13B increased the protein expression of *$p27^{kip}$* by 2 folds at basal levels, and Wnt13A showed more effect in increasing mRNA expression of *$p27^{kip}$* at basal levels than other isoforms, implying that Wnt13A may be related to cell quiescence.

6.4.4.2 Oxidative stress resistance-related genes

Another subset of FOXO target genes is oxidative stress resistance-related *SOD2* and *catalase*, and they are associated with the function of FOXOs in stress resistance. As shown in Figure 6.9A, Wnt13A, Wnt13B and M1L-Wnt13B increased the protein expression of MnSOD both at basal levels and after LPS treatment in transient transfection, and M1L-Wnt13B showed bigger effect (2.5 fold at basal levels and 70% after LPS treatment) than other forms. Wnt13 forms also showed a trend in upregulating mRNA levels of MnSOD at the basal levels, and M1L-Wnt13B had the statistical significance. In the experiments of stable transfection (Figure 6.10), Wnt13A had slightly higher increase in MnSOD protein at basal levels than other forms, and a similar phenomenon was also seen in mRNA levels of MnSOD: 5 fold increase by Wnt13A

while 2-3 fold by other isoforms. These results suggest that Wnt13 forms increased the expression of MnSOD with differential strengths.

As for catalase, the intracellular Wnt13 forms increased the protein levels of catalase by 60-80% at basal levels in transient transfection, and M1L-Wnt13B had stronger effect (Figure 6.11A). But the Wnt13 isoforms did not show significant effect in increasing mRNA expression of catalase (Figure 6.11B). In stable transfection (Figure 6.12), the protein levels of catalase were not obviously upregulated by Wnt13 forms either at basal levels or upon LPS treatment except M74L-Wnt13B, other Wnt13 forms increased mRNA levels of catalase by 2-3 fold, and M1L-Wnt13B was statistically significant ($p<0.05$). So Wnt13 forms, especially nuclear forms, increased catalase expression mainly at protein levels.

6.4.5 Effect of Wnt13 forms on FHRE-luciferase activity in BAEC

As Wnt13 forms decreased FOXO phosphorylation and increased their nuclear localization, we then studied the effect of Wnt13 forms on FOXO transcription activity by using FHRE-luc Reporter construct. Forkhead Responsive Element (FHRE) is the DNA element to bind FOXO factors in FasL promoter, the nucleotide sequence for FHRE is “TAAATAA” (Brunet A et al. 2002), which has one nucleotide difference than the core sequence of canonical FOXO site T/A AAAC A/C” (Biggs WH et al. 2001). The FHRE-luc reporter construct we used was provided by Addgene, which carries a small region of Fas Ligand promoter containing three canonical copies of FHREs (Brunet A et al. 1999).

As shown in Figure 6.13A, either FOXO1-WT or FOXO3a-WT was unable to induce FHRE luciferase activity, so we then used constitutive-activated (CA) or dominant-negative (DN) forms of FOXOs as positive or negative controls to test our system. CA form FOXO3a-TM contains three mutations at phosphorylation sites of Akt, which increased FHRE luciferase activity by 50%, and FOXO3a-H213R is a DN form due to the mutation at DNA binding domain, which reduced FHRE-luciferase activity by 50%; interestingly, FOXO1-AAA did not show any increasing effect on this luciferase activity, suggesting that the FHRE luciferase system was responsive to FOXO3a activation but not FOXO1.

When co-transfected with GFP-FOXO1 or HA-FOXO3a, Wnt13A was able to increase FHRE luciferase activity by 60% or 30%, respectively, but M1L-Wnt13B did not affect the transcriptional activity of exogenous FOXOs (Figure 6.13A). And this phenomenon is inconsistent with the result of FOXO3a localization: M1L-Wnt13B increased the nuclear localization of exogenous FOXO3a more than Wnt13A. Perhaps for FHRE, even though M1L-Wnt13B pushed exogenous FOXO3a into the nucleus, this nuclear FOXO3a-WT was not able to transactivate FHRE, which is supported by the fact that only active form of FOXO3a, not FOXO3a-WT increased the FHRE luciferase activity. For endogenous FOXO activity, none of Wnt13 forms were able to increase the FHRE luciferase activity either at basal levels (Figure 6.13B) or after LPS treatment (data not shown). Therefore, although Wnt13A increased exogenous FOXO transcription activity at FHRE, Wnt13 forms were unable to increase endogenous FOXO transcription activity at FHRE.

6.4.6 Effect of Wnt13 forms on *SOD2* transcriptional regulation in BAECs

6.4.6.1 *SOD2* promoter region

Since MnSOD expression was upregulated by Wnt13 forms at both mRNA levels and protein levels, we tried to determine whether Wnt13 forms function through increasing FOXO activity to promote *SOD2* gene transcription. First, we used the 3 constructs with different lengths of fragments from human *SOD2* promoter region: P7, 1.6K, and 3K (Figure 6.14). And the DNA binding element (DBE) of FOXOs is at -1,249 bp, so if FOXO transcription activity is increased, the luciferase activity of 1.6K or 3K should be greatly enhanced compared to P7. According to Figure 6.15A, without the co-transfection of exogenous FOXOs, Wnt13A had a 1.4 fold increase in P7 than PCR3 control, but not further increase when 1.6K and 3K luciferase constructs were used, suggesting that Wnt13A increase SOD2 gene transcription not through FOXO factors. Moreover, other Wnt13 forms including M1L-Wnt13B did not show obvious effect on the luciferase activity of 1.6K or 3K, indicating that intracellular Wnt13 forms did not enhance SOD2 transcription at promoter region.

Also, as shown in Figure 6.15B, exogenous FOXO1-WT or FOXO3a-WT were unable to increase the luciferase activity of 1.6K. So FOXO activated forms, FOXO3a-AAA and FOXO3a-TM, were used to test the system. And exogenous FOXO3a-AAA and FOXO3a-TM increased the PGL3-1.6K-Luciferase activity by 2 fold or 4 fold, respectively, indicating that the SOD2 1.6K promoter fragment is responsive to FOXO activation. Also, compared to PCR3, Wnt13A enhanced 1.6K luciferase activity up to 2 fold when co-transfected with FOXO1-WT or FOXO3a-WT, indicating that Wnt13A

may increase exogenous FOXO transcription activity at *SOD2*-1.6K promoter region, which is consistent with the result that Wnt13A increased exogenous FOXO transcription activity at FHRE. However, M1L-Wnt13B did not show any increasing effect on either exogenous or endogenous FOXO transcription activity at *SOD2* 1.6K region.

Then we investigated whether Wnt13 forms, especially M1L-Wnt13B, affect *SOD2* promoter luciferase activity upon LPS treatment. Compared to the luciferase activity at basal levels, the activity of PGL3 vector, PGL3-P7 and PGL3-1.6K constructs was not induced by LPS challenge. As shown in Figure 6.16A and 6.16B, the luciferase activity of PGL3 vector, PGL3-P7 and PGL3-1.6K showed a small increase by transient transfection of Wnt13A after LPS treatment, which is similar to the result at basal levels. M1L-Wnt13B had a trend in increasing in luciferase activity of PGL3-P7 and PGL3-1.6K but not PGL3 vector after LPS treatment (Figure 6.16 B and C). Therefore, our results suggest that Wnt13A increase *SOD2* transcription at promoter region at basal levels and after LPS treatment independent of FOXOs; M1L-Wnt13B had a very limited effect on increasing *SOD2* transcription at promoter region.

6.4.6.2 Intron 2 region

Since the promoter region of *SOD2* gene can not fully explain the increased *SOD2* mRNA levels by Wnt13 forms, especially M1L-Wnt13B, we then tested whether the intron2 element (I2E), which are responsive to stress stimuli including TNF and IL-10 because of the NF-κB site, could be regulated by Wnt13 forms. At the basal levels (Figure 6.17A), all the Wnt13 forms except Wnt13B showed slight increase in PGL3-P7-

I2E-luciferase activity, but this was not significant. However, after LPS treatment, the activity of PGL3-P7-I2E was greatly induced (up to 12 fold) compared to that at basal levels, which is expected due to the existence of NF-κB site in the intron 2 region that is responsive for stress stimuli. Moreover, Wnt13A increased PGL3-P7-I2E activity by 50% at basal levels (Figure 6.17A), which is similar to the increase shown in PGL3-P7 and PGL3-1.6K, indicating that this increase may be more from the common fragment P7; and after LPS treatment, Wnt13A did not have further increase in PGL3-P7-I2E activity than basal levels, further suggesting that Wnt13A increases *SOD2* transcription activity mainly through P7 fragment instead of I2E. M1L-Wnt13B enhanced PGL3-P7-I2E-luciferase activity only by 30% at basal levels; however, this increase went up to 2 fold ($p<0.05$) after LPS treatment, which explains a similar increase of *SOD2* mRNA levels upon LPS treatment (Figure 6.9). This result suggests that M1L-Wnt13B increases *SOD2* transcription activity mainly through I2E, especially when challenged by LPS.

In order to find out which site in I2E is responsible for the increase by M1L-Wnt13B dependent of LPS, we subsequently looked into the sequence of I2E for FOXO site. And, an "AAACA" was found from +2053 though + 2057, which is exactly the same as the core sequence of FOXO binding site. To test whether this putative FOXO site was responsive to both FOXO and M1L-Wnt13B, our labatory mutated this putative FOXO site "AAACA" to "AAAGA", and made two maxipreps, I2E-M1, and I2E-M2. As shown in Figure 6.18A, PGL3-P7-I2E luciferase activity at basal levels was induced by the transfection of FOXO3a-TM, a constitutively activated form of FOXO3a by 3 fold. Compared to wildtype PGL3-P7- I2E, the basal luciferase activity of PGL3-P7-I2E-M1 and -M2 was increased by 60% ($p<0.05$) and 30% ($p<0.07$), respectively. And FOXO3a-

TM increased the luciferase activity of PGL3-P7-I2E-M1 and -M2 by 1.5 and 1.3 fold, respectively, which is less than the increase in I2E wildtype. After the treatment with LPS, the PGL3-P7-I2E luciferase activity was induced by 10 fold than that of basal levels, and transfection of FOXO3a-TM increased the activity by 1.2 fold. Also compared to wildtype PGL3-P7- I2E, the basal luciferase activity of both PGL3-P7-I2E-M1 and -M2 was increased by 1 fold. But FOXO3a-TM showed much less increase of luciferase activity of these two mutants by 50% and 20%, respectively than I2E wildtype, showing mutation at this FOXO site can, at least partially, diminish the increase of PGL3-I2E-P7 luciferase activity dependent of LPS. All these data support that the putative FOXO site may be functional.

Then we questioned whether this putative FOXO site is responsible for the increased I2E activity by Wnt13 forms. Figure 6.18C demonstrated that the increase of PGL3-P7-I2E luciferase activity by Wnt13 forms, especially by M1L-Wnt13B, was totally abolished through mutation of the FOXO site located at I2E. Therefore, this putative FOXO site is important for M1L-Wnt13B function, and M1L-Wnt13B enhanced PGL3-P7-I2E luciferase activity after LPS treatment possibly through this novel FOXO site at I2E, suggesting that M1L-Wnt13B may increase *SOD2* transcription after LPS treatment mediated by FOXOs.

6.5 Discussion

FOXOs are a family of critical transcriptional integrators for the homeostasis of endothelial cells (Potente M et al. 2005). FOXO1 deficiency is embryonic lethal for mice

due to branchial arch defects and abnormal vascular remodeling in the yolk sacs (Furuyama T et al. 2004), and FOXO1, 3, 4 triple-knockout mice developed thymic lymphomas and hemangiomas (endothelial cell tumor), suggesting that FOXOs are specifically important for endothelial cell homeostasis (Paik JH et al. 2007). In HUVECs, FOXO1 and FOXO3a are the most abundant FOXO isoforms, and overexpression of constitutively active Foxo1 or Foxo3a significantly inhibits endothelial cell migration and tube formation in vitro (Potente M et al. 2005). Therefore, in our BAEC system, we focused on studying FOXO1 and FOXO3a, and we found distinct features between these two isoforms in BAECs: 1) FOXO1 is more abundant than FOXO3a; 2) for the subcellular localizations, the endogenous FOXO1 was exclusively found in the nucleus but not the cytoplasm (Figure 6.6C) whereas the endogenous FOXO3a was more evenly distributed to each fraction. The exogenous FOXO1 was also more localized in the nucleus (50%) than in the cytoplasm (30%) (Figure 6.4), whereas more FOXO3a is distributed in the cytosol (50%) than in the nucleus (40%) (Figure 6.5). 3) For transcription activity, the FOXO3a-TM (constitutively active form of FOXO3a) is more active than the FOXO1-AAA (constitutively active form of FOXO1), since FOXO3a-TM increased FHRE luciferase activity (by 60%) more than FOXO1-AAA (Figure 6.13), and FOXO3a-TM enhanced SOD2-1.6K luciferase activity (up to 4 fold) more than FOXO1-AAA did (by 2 fold) (Figure 6.15). These data suggest that although both endogenous and exogenous FOXO1-WT is more abundant in BAECs and more FOXO1 stays in nucleus, the promoter assay results did not show FOXO1 is more active than FOXO3a.

In this study, we have also shown the differential effect of Wnt13 forms on FOXOs in BAECs. In transient transfection, nuclear M1L-Wnt13B increased FOXO3a

expression both at mRNA levels and protein levels, but had no significant effect on FOXO3a phosporylation at Ser253, which is in agreement with the unaffected phosphorylation of Akt by M1L-Wnt13B; M1L-Wnt13B increased the nuclear localization of both exogenous FOXO1 and exogenous FOXO3a; M1L-Wnt13B upregulated the expression of other FOXO target genes, such as $p27^{cip}$ (mRNA), MnSOD (mRNA and protein), catalase (protein), which is in agreement with our hypothesis, so M1L-Wnt13B may increase the expression and activation of FOXO3a. However, Wnt13A decreased FOXO3a phosphorylation by 60% but did not increase FOXO3a expression significantly; Wnt13A increased exogenous FOXO1 nuclear localization; Wnt13A increased the nuclear localization of endogenous FOXO3a more than M1L-Wnt13B did, which is in agreement of the increased luciferase activity of FHRE (with exogenous FOXOs) and *SOD2* promoter; and Wnt13A increase the expression of FOXO target genes, like p27 (protein), MnSOD (mRNA and protein), indicating that Wnt13A might also increase FOXO activation. Altogether these results suggest that Wnt13A increased the nuclear localization of FOXO3a through decreasing FOXO3a phosphorylation while M1L-Wnt13B increased the nuclear localization of FOXO3a via upregulating the expression of FOXO3a.

Although both Wnt13A and M1L-Wnt13B promote FOXOs to stay in nucleus, they differ in their effect on FOXO transcription activity. Wnt13A did enhance SOD2 promoter activity, which explains the increased levels of MnSOD, but this effect was not through FOXO site, because there was no obvious difference between SOD2-P7 activity (40% increase) and SOD2-1.6K activity (50% increase) by Wnt13A. So Sp1 or AP-2 sites may be involved in the increased P7 luciferase activity by Wnt13A. And a bigger

increase was seen in SOD-3K luciferase activity (2 fold) compared to SOD2-1.6K, implying that some other element(s) located between 1.6K and 3K is important for SOD2 promoter activity. However, M1L-Wnt13B did not show any effect on *SOD2* luciferase activity of its promoter region; yet, M1L-Wnt13B enhanced *SOD2* luciferase activity through its intron 2 element possibly mediated by a novel FOXO-binding site. Similar differences were also described in the previous chapter that Wnt13A and M1L-Wnt13B increase EC apoptosis and Bim expression with different activity: M1L-Wnt13B displayed robust effect while Wnt13A had mild effect. Therefore, even though both Wnt13A and M1L-Wnt13B increased the FOXO nuclear localization, M1L-Wnt13B seems to exert bigger effect on FOXO activity (at least for SOD2) and expression as well as EC apoptosis while Wnt13A appears to have no significant increase in FOXO activity and in apoptosis induction.

Both the FHRE of *FasL* gene and *SOD2* promoter contain FOXO responsive elements, but none of Wnt13 forms (except Wnt13A) were able to increase luciferase activity of FHRE and *SOD2* promoter (Figure 6.13). So we then looked at SOD2 region known to be regulated by LPS, i.e. intron 2 element which has reported to be induced up to 6 fold by TNF/IL-10 due to the existing NF-κB site (Xu Y et al. 1999). Surprisingly, in our experiment, the PGL3-P7-I2E was also responsive to FOXO3a-TM because the SOD2-P7-I2E luciferase activity at basal levels was induced by FOXO3a-TM up to 4 fold; and after LPS treatment, FOXO3a-TM further induced SOD2-P7-I2E luciferase activity up to 2 fold. Therefore, we started to test if there was a consensus binding site, and we did find one located at +2053 - + 2057 at intron 2 element. Compared to the sequences of the FOXO sites in the other constructs ("TAAATAA" for FHRE and

"TAAACAA" for SOD2 promoter) (Brunet A et al. 2002; Kops GJ et al. 2002), the sequence of this FOXO at I2E is "AAAACAA", which fits with the canonical conserved FOXO binding site "T/A AAAC A/C" (Biggs WH et al. 2001). To test if this FOXO site is functional, two mutants were made, and our results showed that by mutating the putative FOXO site, the 4-fold increase of SOD2-P7-I2E luciferase activity by FOXO3a-TM went down to 2.2 fold at basal levels, and after LPS treatment, the 2-fold increase by FOXO3a-TM were diminished to 20-50% (Figure 6.18), but the big standard errors in FOXO3a-TM groups due to the small size of data (n=2), abolished the significance of the increase by FOXO3a-TM. These findings support the possibility of a novel FOXO-responsive site located in SOD2-I2E region.

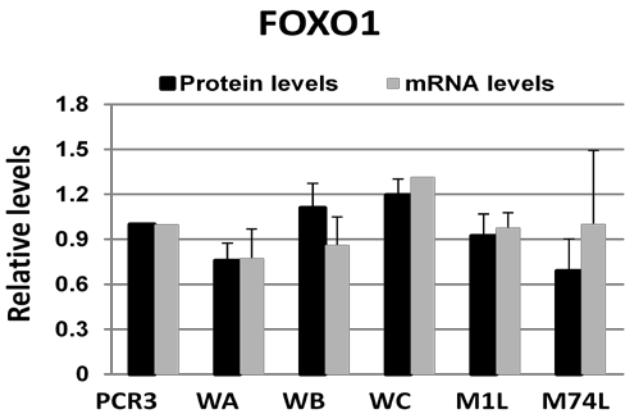
At basal levels, Wnt13 forms had a mild increase (20-30%) in SOD2-P7-I2E luciferase activity; after LPS treatment, this increase went up (2 fold for M1L-Wnt13B) (Figure 6.17). Figure 6.8 demonstrated that the increase in SOD2-P7-I2E luciferase activity (LPS treated) by M1L-Wnt13B, was absent when the putative FOXO site was mutated, suggesting that the putative FOXO site is essential for the increasing effect of M1L-Wnt13B on SOD2-P7-I2E activity. Compared to the DBE site of FOXOs (-1,249 bp) at SOD2 promoter region which was not responsive to M1L-Wnt13B, the novel FOXO site at I2E region is responsible for the increased PGL3-P7-I2E luciferase activity by M1L-Wnt13B. Therefore, M1L-Wnt13B upregulated *SOD2* expression at transcription levels possibly through the FOXO site at I2E.

Also, the increase of PGL3-P7-I2E luciferase activity by M1L-Wnt13B was higher than the increase at basal levels. However, the effect of M1L-Wnt13B on upregulating of the expression of SOD2 as well as other pro-apoptotic factors (caspase-3,

Bim, etc) is similar between basal levels and LPS treatment. Also, Wnt13A increased *SOD2* transcription and expression at basal levels independent of FOXOs. Thus, there may be other mechanisms besides FOXOs are responsible for the effect of Wnt13A and M1L-Wnt13B at basal levels.

Besides *SOD2*, is the effect of Wnt13 forms on Bim also through FOXOs? In endothelial progenitor cells (EPCs), FOXO4 overexpression was shown to increase Bim promoter activity and Bim expression, resulting in increased EPC apoptosis (Urbich C et al. 2005); also in EPCs, silencing FOXO3a led to reduced Bim expression and less cell apoptosis (Zhu S et al. 2008), indicating the regulating role of FOXOs in Bim expression and subsequent cell apoptosis in EPCs. However, it is uncertain whether the effect of FOXOs on Bim regulation in EPCs can extend to differentiated BAECs, so whether Wnt13 forms, especially nuclear Wnt13 forms upregulated Bim expression also through increasing FOXO transcriptional activity still remains to be determined. It seems that Wnt13 forms have distinct effect on each specific target gene of FOXOs: no increase at all in FHRE of FasL gene; no increase by M1L-Wnt13B in SOD2 promoter region, while FOXO-dependent increase by M1L-Wnt13B in SOD2 I2E. So it is very difficult to predict whether Wnt13 forms function also through FOXOs to regulate Bim transcription in BAECs, unless Bim promoter is used.

A)



B)

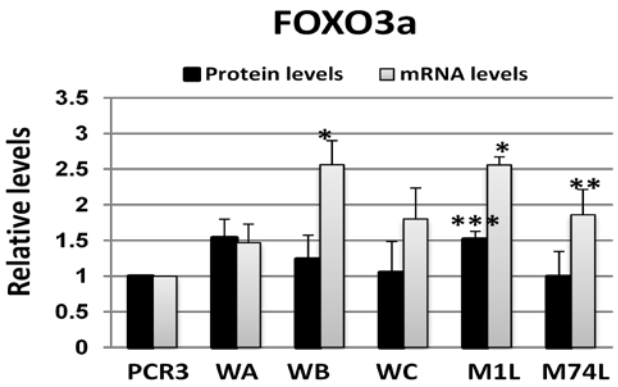


Figure 6.1 Effect of Wnt13 forms on the expression of FOXOs in transient transfection: A) FOXO1 and B) FOXO3a. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 40 hours, and cells were then harvested for immunoblotting with anti-FOXO1 and anti-FOXO3a antibodies or for RNA extraction and real-time PCR. The relative levels after normalization with basal PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6-15 independent transfection experiments; *<0.05, **<0.01, ***<0.001).

A) B)

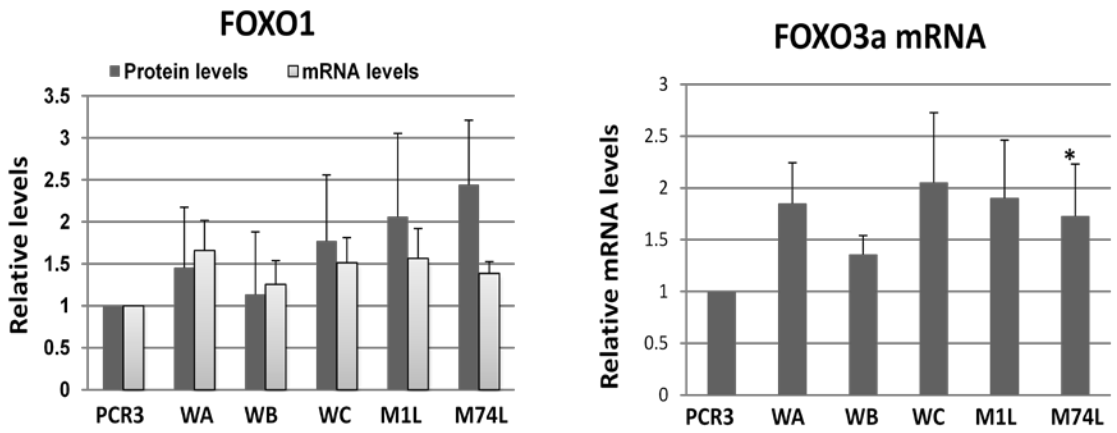
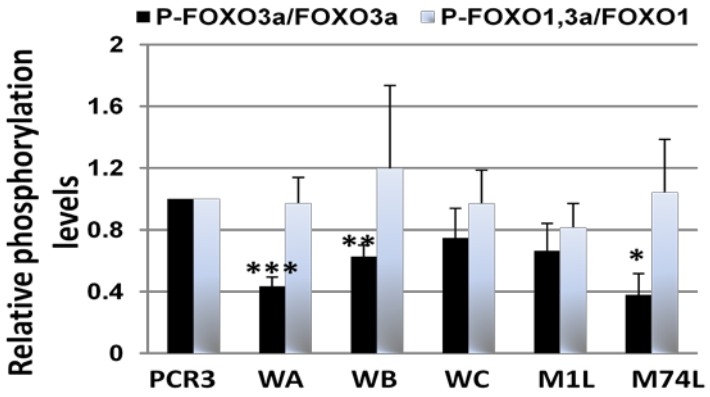


Figure 6.2 Effect of Wnt13 forms on FOXO expression in stable-transfected BAECs: A) FOXO1; B) FOXO3a. BAECs were stably transfected with the PCR3 vector or Wnt13-Flag constructs, and cells were then harvested for the immunoblotting with anti-FOXO1 antibody or for RNA extraction and real-time PCR. The relative levels after normalization with basal PCR3 values (set as 1) are represented in the graph (mean±SEM, n=5-6 independent transfection experiments; *<0.05).

A)



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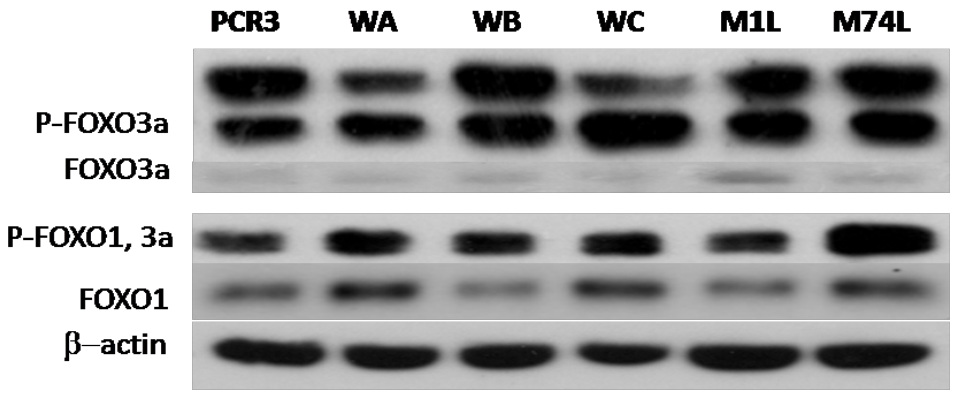
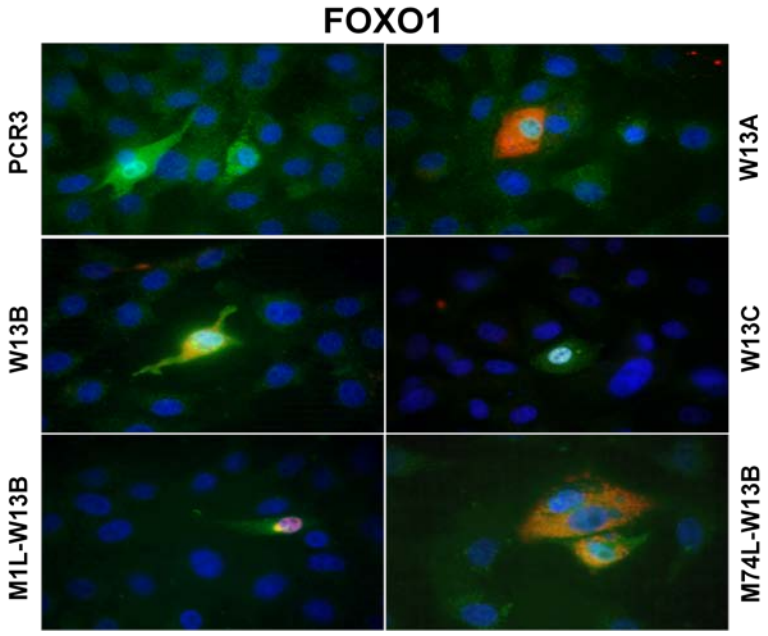


Figure 6.3 Effect of Wnt13 forms on FOXO phosphorylation in transiently-transfected BAECs. A) BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 40 hours, and cells were then harvested for immunoblotting with P-FOXO1 (Thr24)/FOXO3a (Thr32), P-FOXO3a (Ser253) and FOXO1 as well as FOXO3a antibodies. The relative phosphorylation levels after normalization with basal PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6-15 independent transfection experiments; *<0.05, **<0.01, ***<0.001). B) Representative images of western blotting analysis are shown.

A)



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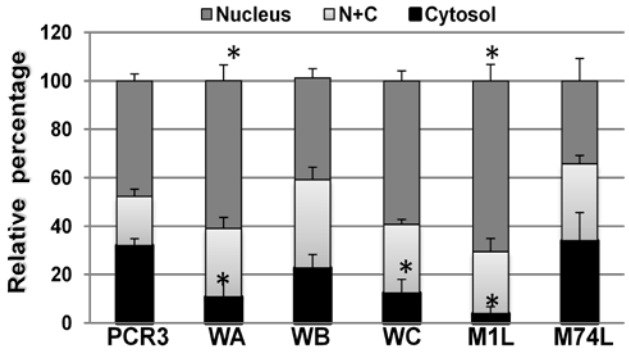
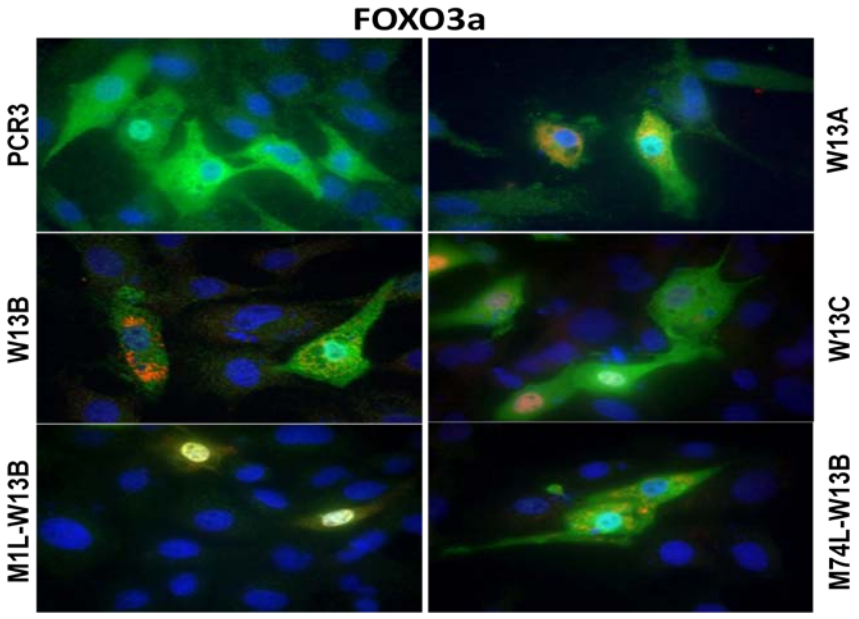


Figure 6.4 Effect of Wnt13 forms on subcellular localizations of exogenous FOXO1 in transiently-transfected BAECs. BAECs were co-transfected with Wnt13-Flag constructs and GFP-FOXO1 for 40 hours, and then fixed with 4% formaldehyde, permeabilized in 0.1% Triton, and stained with rabbit polyclonal anti-Flag (red) and mouse monoclonal anti-GFP (green) antibodies, followed by the incubation of Alexa goat anti-rabbit 488 and

Alexa goat anti-mouse 568 antibodies. A) Representative images are shown (Red: Wnt13-Flag; green: GFP-FOXO1; blue: DAPI); B) quantification of the relative percentage in the nucleus/nucleus and cytoplasm/ cytoplasm is represented in the graph (mean±SEM, n=5 independent transfection experiments; One Way ANOVA was used for comparison between groups, *<0.05).

A)



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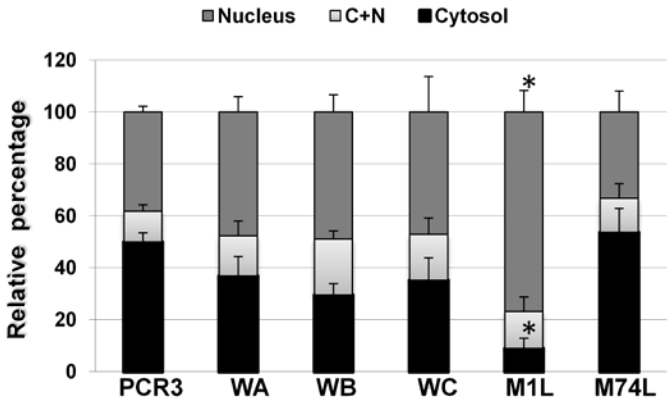
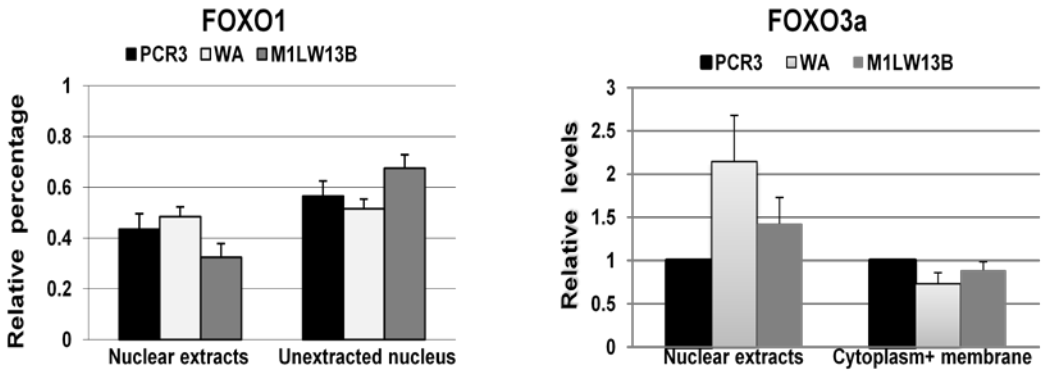


Figure 6.5 Effect of Wnt13 forms on subcellular localizations of exogenous FOXO3a in transiently-transfected BAECs. BAECs were co-transfected with Wnt13-Flag constructs and HA-FOXO3a for 40 hours, and then fixed with 4% formaldehyde, permeabilized in 0.1% Triton, and stained with rabbit polyclonal anti-Flag (red) and mouse monoclonal anti-HA (green) antibodies, followed by the incubation of Alexa goat anti-rabbit 488 and

Alexa goat anti-mouse 568 antibodies. A) Representative images are shown (Red: Wnt13-Flag; green: HA-FOXO3a; blue: DAPI); B) quantification of the relative percentage in the nucleus/nucleus and cytoplasm/ cytoplasm is represented in the graph (mean±SEM, n=6 independent transfection experiments; One Way Anova was used for comparison between groups, *<0.05).

A) B)



C)

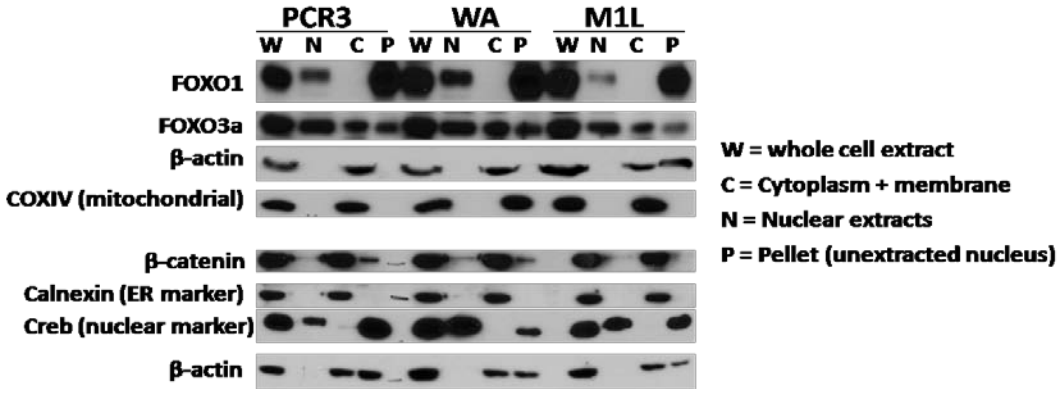
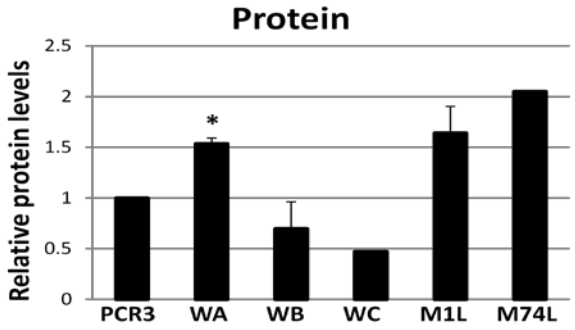


Figure 6.6 Effect of Wnt13 forms on endogenous subcellular localizations of FOXOs in transiently-transfected BAECs. A) BAECs were transfected with Wnt13-Flag constructs for 40 hours, and cell fractions were isolated followed by immunoblotting. A) Quantification of the relative percentage of FOXO1 in each subcellular fraction is represented in the graph; B) quantification of the relative levels of FOXO3a over PCR3 value (set as 1) in each subcellular fraction is represented in the graph (mean±SEM, n=4-5 independent transfection experiments); C) representative images of western blotting analysis is shown.

A)



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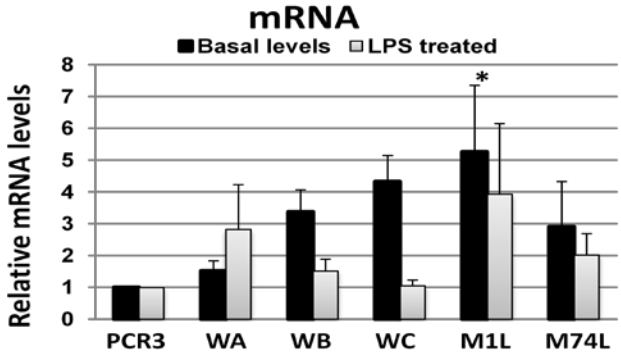


Figure 6.7 Effect of Wnt13 forms on $p27^{kip}$ expression in transiently-transfected BAECs. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 24 hours, followed by 100ng/ml LPS treatment, A) 16 hours later, whole cell exacts were prepared and p27cip and β actin were analyzed by immunoblotting with specific antibodies; or B) 6 hours later, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative protein or mRNA levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=1-6 independent transfection experiments; *<0.05).

A) B)

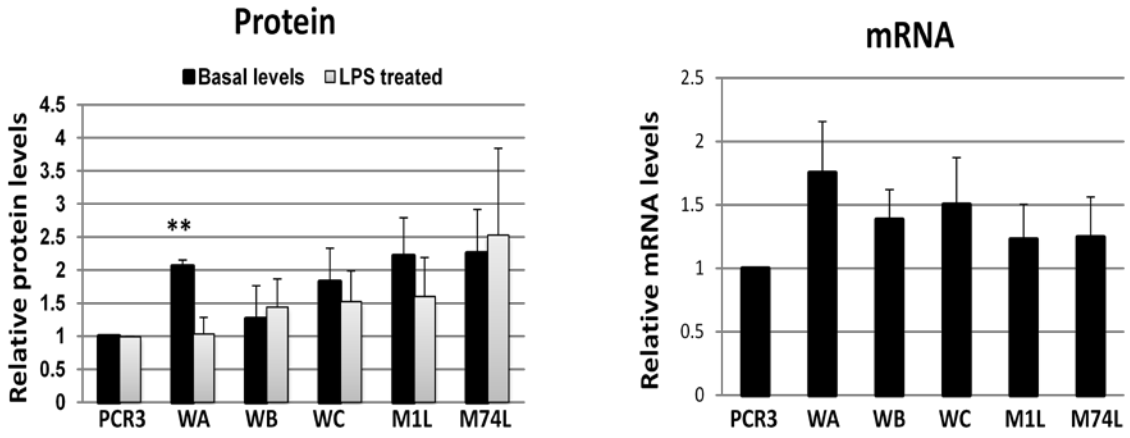


Figure 6.8 Effect of Wnt13 forms on $p27^{kip}$ expression in stably-transfected BAECs. BAECs were stably transfected with the PCR3 vector or Wnt13-Flag constructs, followed by 100ng/ml LPS treatment, A) 16 hours later, whole cell exacts were prepared and p27cip and β actin were analyzed by immunoblotting with specific antibodies; or B) 6 hours later, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative protein or mRNA levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6 independent transfection experiments; *<0.05).

A) B)

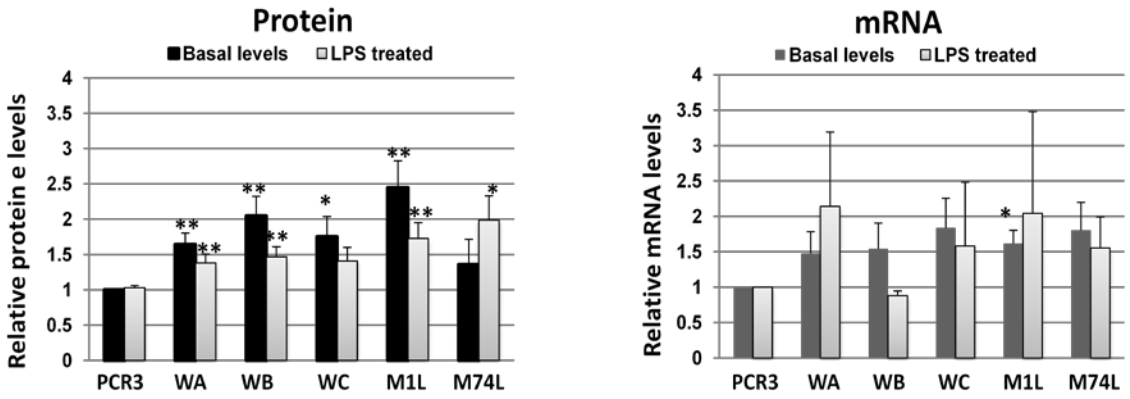


Figure 6.9 Effect of Wnt13 isoforms on MnSOD expression in transiently-transfected BAECs. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 24 hours, followed by 100ng/ml LPS treatment, A) 16 hours later, whole cell exacts were prepared and MnSOD and β actin were analyzed by immunoblotting with specific antibodies; or B) 6 hours later, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative protein or mRNA levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=2-18 independent transfection experiments; *<0.05; **<0.01).

A) B)

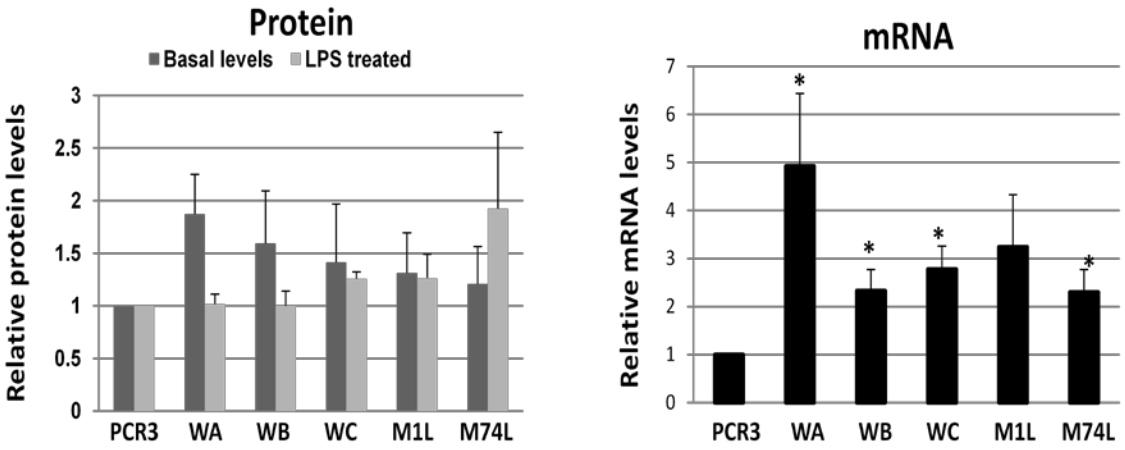


Figure 6.10 Effect of Wnt13 isoforms on MnSOD expression in stably-transfected BAECs. BAECs were stably transfected with the PCR3 vector or Wnt13-Flag construct, followed by 100ng/ml LPS treatment, A) 16 hours later, whole cell exacts were prepared and MnSOD and β actin were analyzed by immunoblotting with specific antibodies; or B) 6 hours later, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative protein or mRNA levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6 independent transfection experiments; *<0.05).

A)

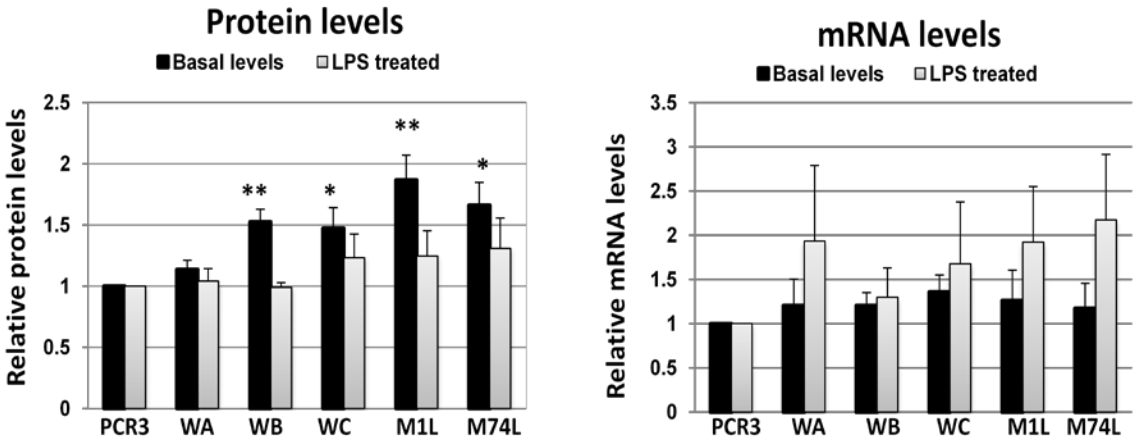


Figure 6.11 Effect of Wnt13 isoforms on catalase expression in transiently-transfected BAECs. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs for 24 hours, followed by 100ng/ml LPS treatment, A) 16 hours later, whole cell exacts were prepared and catalase and β actin were analyzed by immunoblotting with specific antibodies; or B) 6 hours later, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative protein or mRNA levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=2-10 independent transfection experiments; *<0.05; **<0.01).

A) B)

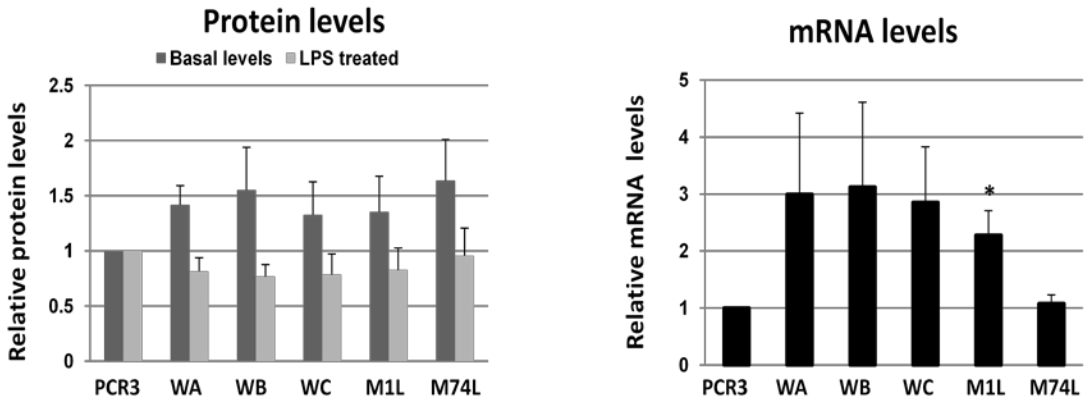
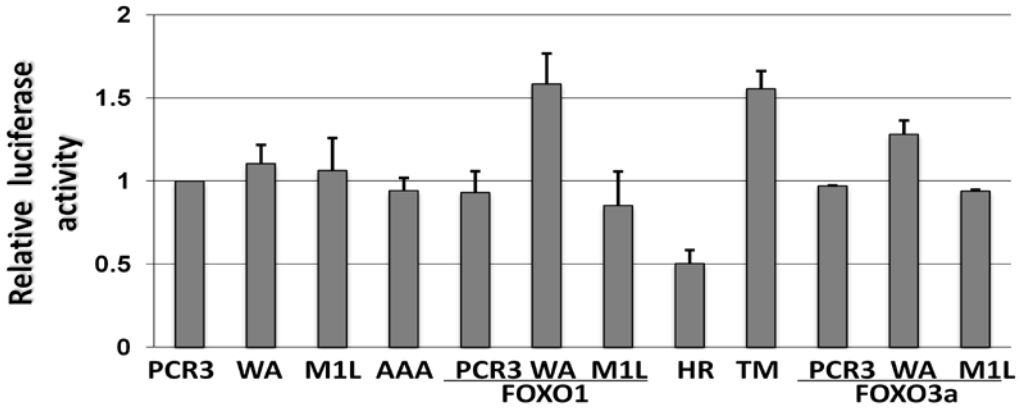


Figure 6.12 Effect of Wnt13 isoforms on catalase expression in stably-transfected BAECs. BAECs were stably transfected with the PCR3 vector or Wnt13-Flag constructs, followed by 100ng/ml LPS treatment, A) 16 hours later, whole cell exacts were prepared and catalase and β actin were analyzed by immunoblotting with specific antibodies; or B) 6 hours later, cells were harvested and treated with Trizol prior to RNA extraction and real-time PCR analysis. The relative protein or mRNA levels after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=6 independent transfection experiments; *<0.05).

A)



B)

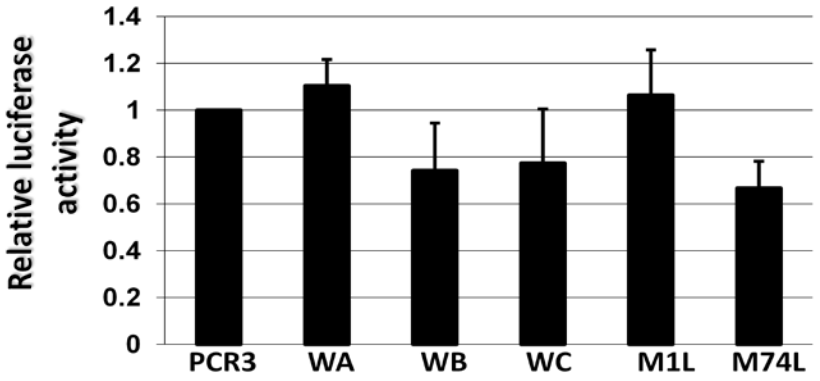


Figure 6.13 Effect of Wnt13 forms on FHRE-luciferase activity in transiently-transfected BAECs: A) exogenous FOXO activity; B) endogenous FOXO activity. BAEC were transiently transfected with the PCR3 vector or Wnt13-Flag constructs and FHRE luciferase construct together A) with or B) without different forms of exogenous FOXO1 or FOXO3a for 40 hours, followed by luciferase activity assay. The relative luciferase activity after normalization with basal PCR3 values (set as 1) are represented in the graph (mean±SEM, n=2-8 independent transfection experiments).

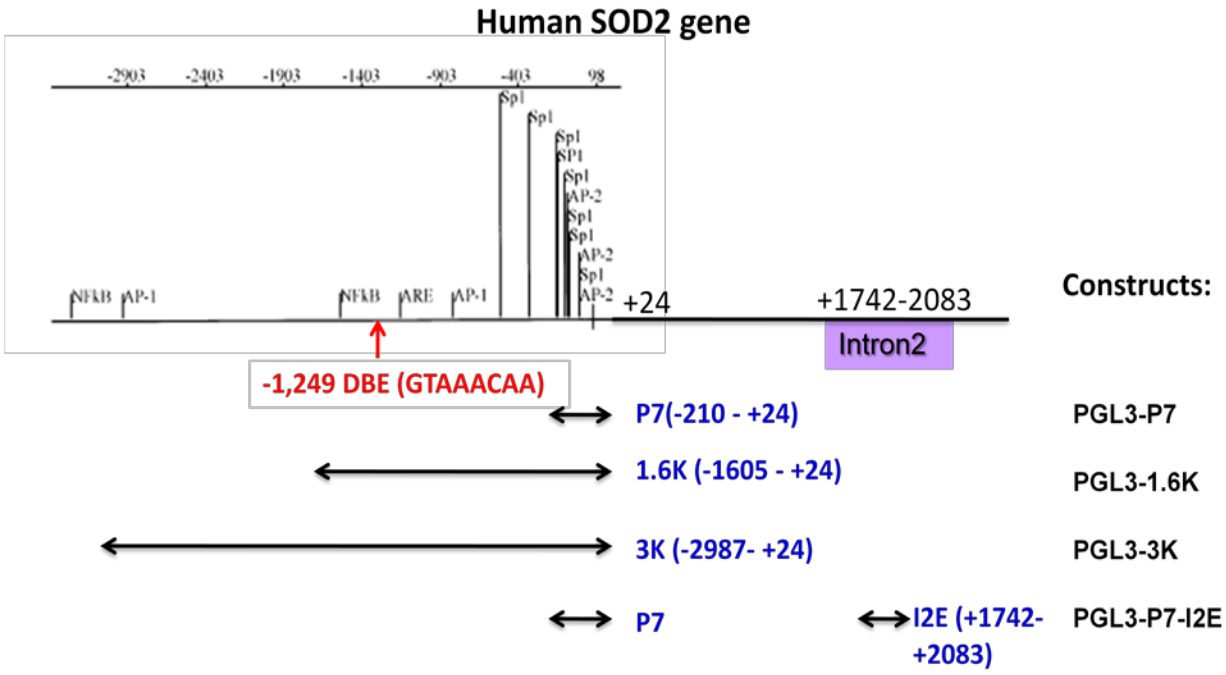
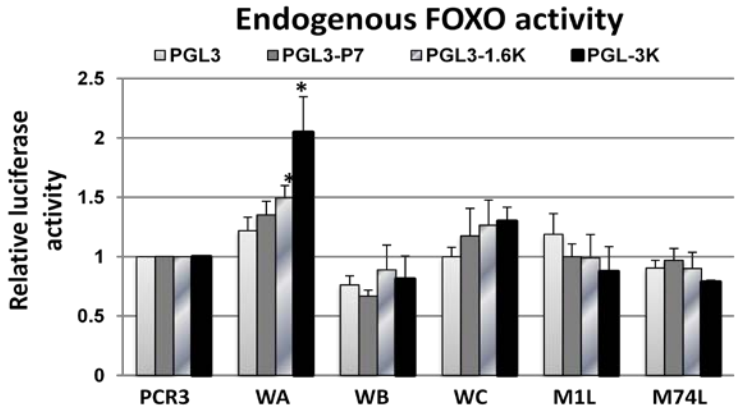


Figure 6.14 Schematic representations of the promoter region and intron region in human *SOD2* gene and the constructs used in this chapter. In human *SOD2* gene, the DNA binding site (DBE) of FOXO is located at -1,249. Three constructs with fragments (P7, 1.6K, 3K) from the promoter region were used, and 1.6K as well as 3K contain the FOXO DBE. The intron 2 element (+1742-+2083) contains a NF-κB site, and the corresponding construct is PGL3-P7-I2E (modified from Xu Y et al. 2002 Figure 1).

A)



B)

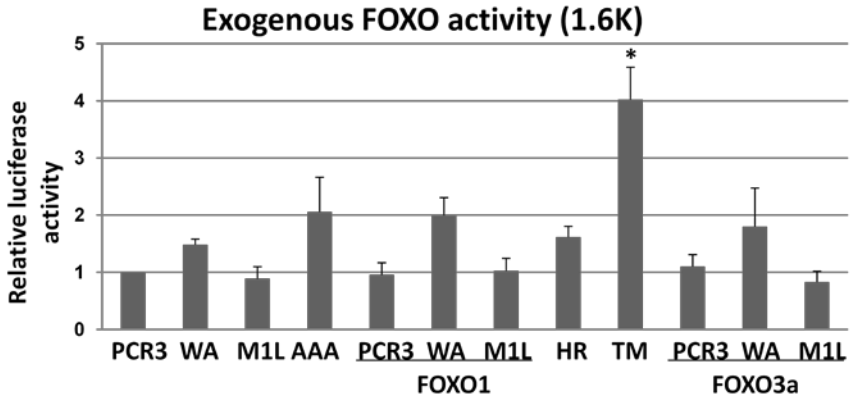
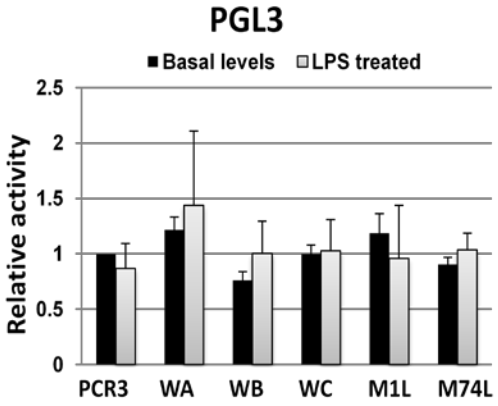
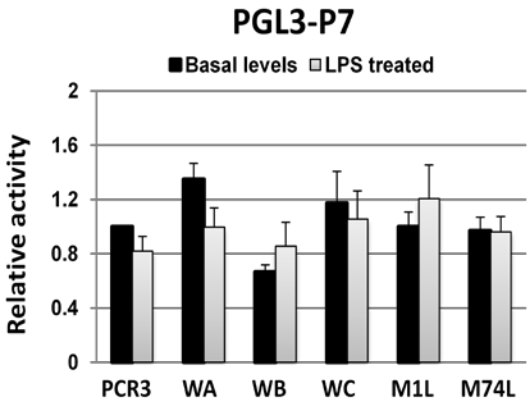


Figure 6.15 Effect of Wnt13 forms on *SOD2* promoter luciferase activity at basal levels in BAECs: A) endogenous FOXO activity; B) exogenous FOXO activity. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs and *SOD2*-1.6K-luicferase constructs, together A) with or B) without different forms of exogenous FOXO1 or FOXO3a for 36 hours, followed by luciferase activity assay. The relative luciferase activity after normalization with basal PCR3 values (set as 1) are represented in the graph (mean±SEM, n=3-12 independent transfection experiments; *<0.05).

A)



B)



C)

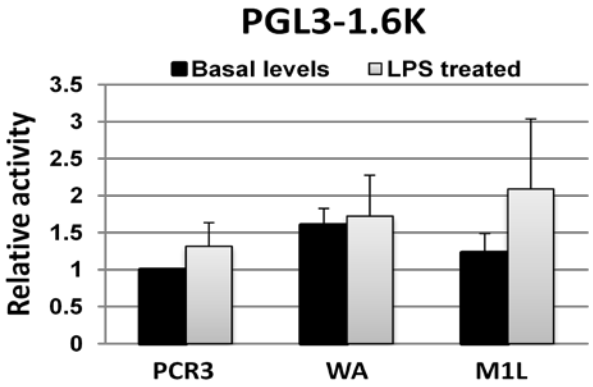


Figure 6.16 Effect of Wnt13 forms on *SOD2* promoter luciferase activity upon LPS treatment in BAECs. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs and different luciferase constructs (A, PGL3 alone; B, PGL3-P7; C, PGL3-1.6K) for 24 hours, followed by LPS treatment (100ng/ml). 12 hours later, the dual luciferase activity assay was performed. The relative luciferase activity after normalization with basal or LPS treated PCR3 values (set as 1) are represented in the graph (mean±SEM, n=2-5 independent transfection experiments).

A) B)

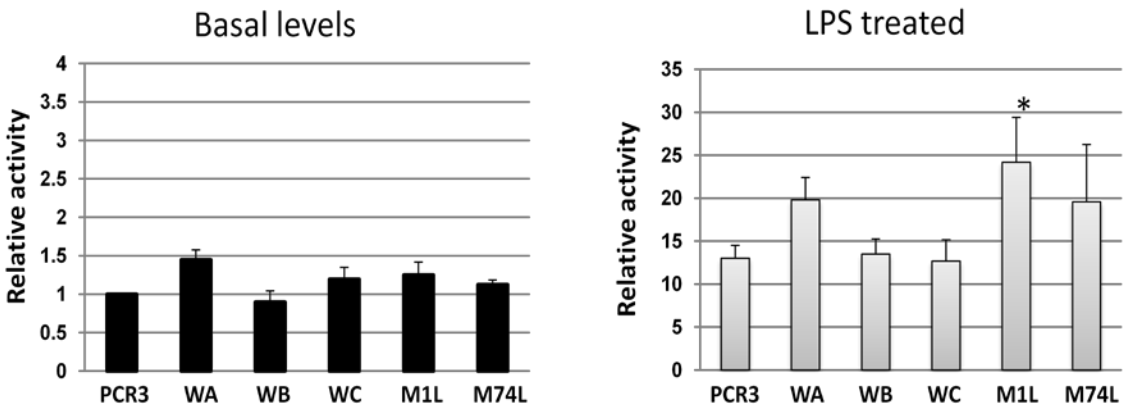
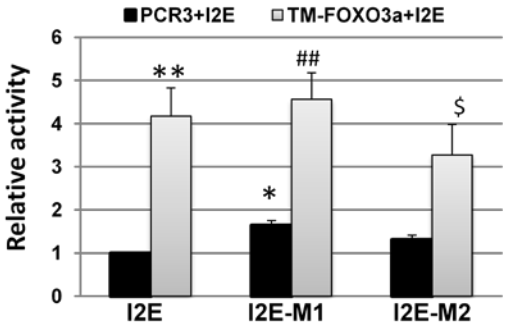
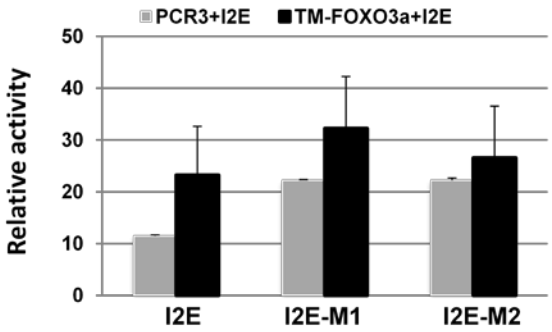


Figure 6.17 Effect of Wnt13 forms on *SOD2* P7- intron2 element (I2E) luciferase activity in BAECs. BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs and PGL3-SOD2-P7-I2E luciferase construct for 40 hours, A) without or B) with the 100ng/ml LPS treatment for 12 hours later, the dual luciferase activity assay was performed. The relative luciferase activity after normalization with basal PCR3 values (set as 1) are represented in the graph (mean±SEM, n=3-10 independent transfection experiments). For the relative luciferase activity after LPS treatment B), One Way ANOVA was used for comparison between groups; *<0.05.

A) Basal levels



B) LPS treated



C) LPS treated

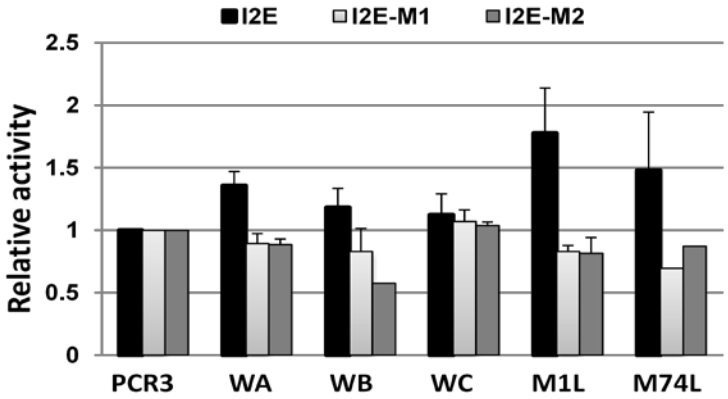


Figure 6.18 Wnt13 forms increased PGL3-P7-I2E luciferase activity after LPS treatment possibly through a putative FOXO site. BAECs were transiently transfected with the PCR3 vector or FOXO3a-TM and different luciferase constructs (PGL3-P7-I2E or PGL3-P7-I2EM1 or PGL3-P7-I2EM2) for 40 hours A) without or B) with LPS treatment for 12 hours, followed by luciferase activity assay. C) BAECs were transiently transfected with the PCR3 vector or Wnt13-Flag constructs and different luciferase constructs (PGL3-P7-

I2E or PGL3-P7-I2EM1 or PGL3-P7-I2EM2) for 24 hours, followed by LPS treatment (100ng/ml). 12 hours later, the dual luciferase activity assay was performed. The relative luciferase activity after normalization with basal PCR3 values (set as 1) are represented in the graph [mean±SEM, n=1-9 independent transfection experiments; *<0.05, **<0.01, compared to PCR3/I2E (basal); ##<0.01, compared to PCR3/I2E-M1 (basal); $<0.05, compared to PCR3/I2E-M2 (basal)]. For Figure 6.17B, One Way ANOVA was used for comparison between groups.

CHAPTER 7. GENERAL DISCUSSION

7.1 Summary

The purpose of this research was to test the hypothesis that in differentiated endothelial cells, 1) the expression of nuclear Wnt13 forms is regulated at translational levels during apoptosis; 2) the nuclear Wnt13 forms favor apoptosis through affecting the activity and/or expression of pro-apoptotic or anti-apoptotic factors; 3) different isoforms of Wnt13 may have differential effects on endothelial cell apoptosis and apoptosis-related factors.

First, since the short form of Wnt13B, Wnt13C and M1L-Wnt13B displayed distinct expression patterns even though they encode the same protein translated form AUG+74, and nuclear Wnt13 forms increased endothelial cell sensitivity to apoptosis (Struewing IT et al. 2006), we were wondering whether the nuclear Wnt13 forms behave like other apoptosis-related factors such as Bcl-2 and c-myc, which undergo translational regulation during apoptosis. Our findings revealed that Wnt13C expression was increased in response to stress and apoptosis-inducers (including MG132), and appeared to be correlated with caspase-3 cleavage. This regulation did not seem to occur at the transcriptional level since there was no significant increase at mRNA level upon the treatment of MG132. And in BAEC, the insertion of Myc tag at the first AUG in Wnt13C mRNA not only inhibited the expression of exogenous Wnt13C at basal level, but also totally abolished the increase of Wnt13C expression by MG132 treatment (Figure 4.3), which indicated the RNA sequences or structures are critical for Wnt13C expression,

suggesting that nuclear Wnt13C can be regulated during apoptosis more likely at the translational levels than the transcriptional levels. Further studies in our laboratory have shown that the translational regulation of Wnt13C is not through internal ribosomal entry, a common cap-independent mechanism by which apoptotic factors initiate their translation during apoptosis, but more likely through upstream open reading frames (Tang T et al. 2008).

Second, the nuclear forms of Wnt13C have been shown to increase endothelial cell susceptibility to apoptosis (Struewing IT et al. 2006), and we confirmed this result by showing the increase in caspase-3 like activity as well as the cleavage of caspase-3 and 7 by the nuclear Wnt13 forms (M1L-Wnt13B) after LPS treatment. The increased cleavage of caspase-3 and 7 by may result, at least in part, from the increased expression of caspase-3 and -7 by M1L-Wnt13B after LPS treatment. M1L-Wnt13B was unable to significantly increase either other inflammatory caspases (caspase-4 and caspase-5) or apoptotic factors (TRAIL, DR5, and caspase8) in the extrinsic apoptotic pathway. However, for Bcl-2 family members, Wnt13 forms were unable to change Bax/Bcl-2 ratio, but upregulated the pro-apoptotic Bim expression.

To discover the underlying mechanisms and possible signaling pathway that the nuclear Wnt13 forms function through, we excluded the activation of Akt or GSK3β as the upstream event that responsible for the increased apoptosis by nuclear Wnt13 forms. We then suspected FOXO transcription factors due to the facts that 1) the nuclear Wnt13 forms may have accessibility to certain transcription factors; 2) both caspase-3 and Bim, which mRNA expression was upregulated by the nuclear Wnt13 forms, are target genes of FOXOs. Subsequently, we demonstrated that nuclear Wnt13 forms upregulated the

expression of FOXO3a but not FOXO1, but the nuclear forms did not reduce the phosphorylation of FOXO1 at Thr24 or FOXO3a at Ser253. And the nuclear forms increased nuclear localization of exogenous both FOXO1 and FOXO3a, implying that enhanced FOXO activation by the nuclear forms. Our data also showed that the nuclear M1L-Wnt13B upregulated the expression of other FOXO target genes such as cell cycle arrest-related *p27* and oxidative stress resistance-related *SOD2*, suggesting the increased FOXO transcription activity by the nuclear Wnt13 forms. Thereby, to confirm that the nuclear Wnt13 forms function via increasing FOXO transcription activity, we used FHRE (Forkhead responsive element from *FasL* promoter) reporter system and the luciferase constructs with *SOD2* promoter or intron 2 for dual luciferase assay. And we showed that nuclear Wnt13 forms were unable to enhance luciferase activity using FHRE luciferase reporter from *FasL* gene and *SOD2* promoter. However, the luciferase activity of SOD2 intron 2 element (I2E) was increased by M1L-Wnt13B upon LPS treatment, which may explain the increased *SOD2* expression by the nuclear Wnt13 forms. Interestingly, a novel putative FOXO site was found in intron 2, which was responsive to activated FOXO3a form (FOXO3a-TM), and found critical for increased I2E luciferase activity by M1L-Wnt13B (Figure 6.18), so M1L-Wnt13B increased SOD2 transcription upon LPS treatment possibly through a putative FOXO site ("AAACA") from +2053 though + 2057 at intron 2 in SOD2 gene. Although we have not confirmed increased FOXO activity at Bim promoter so far, it is still possible that M1L-Wnt13B increase FOXO-mediated Bim transcription though upregulating FOXO3a expression and increasing FOXO nuclear translocation, thereby upregulating Bim expression and tip the balance to pro-apoptosis,

resulting in increased caspase activity and more percentage of apoptotic nuclei in endothelial cells.

Third, considering that Wnt13 isoforms have different subcellular localization, we assumed that Wnt13 forms may have differential effects on endothelial cell apoptosis and the expression/activity of apoptotic regulators. For EC apoptosis, exogenous mitochondrial Wnt13 forms increased cleavage and protein expression of caspase-3, 7 rather than caspase-3 like activity, and secreted Wnt13A increase caspase-3 expression but not caspase-3 like activity or caspase-3 cleavage in BAECs. Therefore our data indicate that compared to nuclear Wnt13 forms, the mitochondrial forms and secreted Wnt13A had weaker action in increasing apoptosis in BAECs, and that the strengths that Wnt13 forms increase apoptosis in BAEC were nuclear (robust) > mitochondrial (moderate) > secreted forms (weak). All the Wnt13 forms did not significantly increase the levels of inflammatory caspases and regulators in extrinsic pathway. For Bcl-2 family members, like the nuclear Wnt13 forms, the mitochondrial and secreted Wnt13 forms did not affect Bax/Bcl-2 ratio but upregulated Bim expression; however, the effect on Bim expression by the mitochondrial or secreted forms was weaker than that by the nuclear forms. Like the nuclear forms, the mitochondrial forms and Wnt13A also did not show increasing effect on the activation of Akt and GSK3β. For FOXOs, unlike the nuclear M1L-Wnt13B, Wnt13A may increase the nuclear localization of endogenous FOXO3a by reducing FOXO3a phosphorylation at Ser253 which is an Akt site rather than upregulating FOXO3a expression. Interestingly, Wnt13A increased *SOD2* expression moderately through *SOD2* promoter region, which was independent on FOXO factors. However, although the mitochondrial Wnt13 forms had a moderate effect on upregulating

FOXO3a levels and decreasing FOXO3a phosphorylation, they did not affect either the nuclear localization of FOXOs or FOXO transcriptional activity using the FHRE-luciferase reporter system, *SOD2* promoter or SOD2 intron 2 element.

Overall, our results suggest that during EC apoptosis, on the one hand, the nuclear Wnt13C is induced at translational level; and on the other hand, the nuclear Wnt13 forms may increase EC susceptibility to apoptosis via upregulating pro-apoptotic factors Bim and caspase-3/7 as well as FOXO3a, which forms a amplification loop further facilitating the completion of apoptotic program in endothelial cells. Besides, mitochondrial Wnt13 forms and Wnt13A have similar but weaker effect on EC apoptosis than nuclear Wnt13 forms do, which appears to be a FOXO-independent event.

7.2 Insights from the regulation of Wnt13C during apoptosis

In Aim1, our results indicated that the nuclear Wnt13C can be regulated during apoptosis more likely at translational levels than transcriptional levels. Under cell apoptosis, while the global rate of translation by classical cap-dependent mechanism is shunt down, alternative translational mechanisms are used to maintain protein synthesis. For example, the pro-apototic protein c-myc (Adachi S et al. 2000), as well as the anti-apoptotic protein Bcl-2 (Sherrill KW et al. 2004) allow cell fate decision towards either apoptosis or survival by employing internal ribosomal entry, which is one of the alternative cap-independent mechanisms. Hence, the presence of internal ribosomal entry site (IRES) in Wnt13C mRNA was firstly suspected to explain the regulation mechanism of Wnt13C. However, using dicistronic Renilla –firefly (FL) luciferase assay, the 5'-UTR

of Wnt13C did not display an IRES activity with or without the treatment of MG132 (Tang T et al. 2008).

Subsequently, other alternative mechanisms such as upstream opening reading frames (uORFs) were considered to be the possible explanation for the translational control of Wnt13C during apoptosis. Further studies in our laboratory suggest that uORFs may be responsible for reducing the translational efficiency in regulation of Wnt13C expression, because 1) the insertion of the 5' leader sequences of Wnt13C into RL reporter led to a 95% and 85% inhibition of translation activity *in vitro*; 2) two uORFs were found in Wnt13C-leader sequences, and the expression of Wnt13C-Flag increased with the deletion of upstream AUG or CUG and the mutation of AUG (+1); 3) the 5' leader sequences of Wnt13B harbor one less uORF than that of Wnt13C, and short Wnt13B showed higher expression than Wnt13C.

The structural features in UTRs are crucial for post-transcriptional regulation in gene expression. It is noteworthy that 5'- UTRs in 50% of human mRNAs and in 35% of mammalian mRNAs contain upstream AUG, and when the uAUG is followed by an in-frame stop codon, it creates an uORF, which is found in around 10% of human or mammalian 5'-UTRs (Mignone F et al. 2002). The presence of uORFs in 5'-UTRs tightly controls the translation of various factors, such as growth factors, cytokines, proto-oncogenes, and the deregulation of uORFs are involved in diseases including melanoma and Alzheimer's disease (Chatterjee S et al. 2009). Moreover, uORFs are also found in the mRNAs of apoptosis-related factors like Bcl-2 (Harigai M et al. 1996), Bax (Salomons GS et al. 1998), and Bim (Gilley J et al. 2005).

A ribosome scanning an mRNA with multiple uORFs may have one of the following fates: 1) it may translate a short peptide (uORF), and remain associated with the mRNA, continuing scanning, resulting in re-initiation at a proximal or distal downstream AUG; 2) it may translate a short peptide (uORF), remaining associated with the mRNA, and then continue scanning until recognize the AUG codon in the uORF in a suboptimal context and scan past it to initiate at a downstream ORF (leaky scanning); 3) it may translate a short peptide (uORF) and then dissociate prematurely at the stop codon; 4) it may be stalled during the elongation or termination phase to block the additional ribosomal scanning, which is mainly mediated by the peptide produced from the uORF through its interaction with some part of the translation machinery (Morris DR et al. 2000). Overall, all these actions that uORFs take will end up inhibiting the translational rate of the mRNA. Additionally, the uORF may also be involved in the destabilization of its own mRNA by a nonsense-mediated mRNA decay (NMD) pathway to affect its gene expression, as evidenced by the uORF in CPA1 mRNA (encoding the small subunit of the arginine pathway carbamoylphosphate synthetase), which is critical for the sensitivity to NMD (Ruiz-Echevarría MJ et al. 2000). So far, it is still unclear which mechanism is responsible for the uORF-dependent regulation in Wnt13C; however, we can propose that the uORFs of Wnt13C 5'-UTR employ to control the mRNA translation possibly through one (or more) of above choices (re-initiation, leaky scanning, ribosomal dissociation, ribosomal stalling) in the fate of mRNA with uORFs during its translation.

7.3 Insights form the increased apoptosis by Wnt13 forms

7.3.1 Possible mechanisms of increased apoptosis by the nuclear Wnt13 forms

In our study, Wnt13 forms, especially the nuclear M1L-Wnt13B, increase caspase-3 like activity and cleavage of caspase-3 and 7 possibly through 1) increased expression of executioner caspase 3 and 7; 2) upregulated expression of the pro-apoptotic Bcl-2 member Bim, suggesting the involvement of the intrinsic apoptotic pathway.

7.3.1.1 Caspase-3 and -7

How the nuclear Wnt13 forms upregulate the expression of pro-caspases has yet to be investigated. The regulation of caspase expression can take place at the transcriptional levels. One factor that influences pro-caspase gene expression is interferon regulatory factor (IRF)-1. Ectopic overexpression of IRF-1 was shown to transactivate the endogenous *ICE/CED 3* gene (former name for inflammatory caspase-1) in lymphocytes (Tamura T et al. 1995). The same results were also seen in human U937 cells which were treated by γ-interferon resulting in the induction of *ICE* expression (Tamura T et al. 1996). In addition, γ-interferon was found to induce the upregulation of caspase 1, 2, 3, 6, 8, and 9 in human erythroid progenitor cells (Dai C et al. 1999). The up-regulation of caspases is associated with cell differentiation in all these models. Besides IRF-1, another transcription factor STAT1 is also required for constitutive caspase expression. In human fibroblasts, STAT1 deficiency (U3A cells) resulted in 10 times lower levels of caspase-1, 2, and 3 at mRNA expression than control cells, and re-introduction of STAT1 into U3A cells were able to restore the expression of caspase-1 mRNA (Kumar A et al. 1997).

The promoter of caspases were also cloned and characterized for further study of the transcriptional regulation of caspases. The rat caspase-3 promoter lacks TATA box but contains a cluster of Sp1 sites, and an Ets-1 binding site is found between -1646 and -1632, which is critical for sustained promoter activity, revealing that Est-1 like transcription factors may regulate the transcription of rat caspase-3 (Liu W et al. 2002). Hypoxia-inducible factor (HIF)-1 was shown to be increased with the same pattern as pro-caspase-3 in vivo after ischemia, and gel shift assay demonstrated a specific HIF-1 binding activity to the promoter of caspase-3, suggesting that HIF-1 may regulate caspase-3 expression at the transcriptional levels (Van Hoecke M et al. 2007). The sequence of human caspase-3 promoter shares 60% homology with that of rat promoter: the similarity is the absence of TATA box and the presence of Sp-1 like sequences; however, the human caspase-3 promoter is shown to be activated by not only Sp1, but also p73 isoforms instead of p53, both (Sp1 and p73) mediated by Sp-1 like sequences (Sudhakar C et al. 2008). The murine caspase-3 promoter was also cloned and identified, with the finding of putative transcription factor binding sites, including elements of NFAT, E2F1, Myc, and p53 (Sabbagh L et al. 2006), but more studies are needed to confirm which transcription factor is involved in the regulation of murine caspase-3 transcription. FOXO1 was found to activate caspase-3 transcription using mouse caspase-3 promoter, which is mediated by direct binding of FOXO1 to the upstream regulatory sequences (Bois PR et al. 2005). As for the nuclear Wnt13 forms, increased caspase-3 expression include both mRNA levels and protein levels, so it is more likely that the upregulation of caspase-3 expression by the nuclear Wnt13 forms occurs at the transcriptional levels. It is still possible that the nuclear Wnt13 increased caspase-3

expression through FOXOs, which needs to be confirmed in caspase-3 promoter assay. Otherwise, other mediators, such HIF-1, STAT-1 and p73, might be responsive for increased transcription of caspase-3 by nuclear Wnt13 forms.

For caspase-7, its protein levels were induced in response to statin treatment in dose-dependent manner, and CHIP assay demonstrated that SREBP-1 and -2 could bind to the proximal promoter region of *caspase-7* gene, whose expression was suppressed by silencing SREBP-1 or -2 (Gibot L et al. 2009). In addition, another study showed that human caspase-7 promoter contained a putative p53-binding site and its activity could be induced by p53 (Joshi B et al. 2007). Therefore, the upregulated caspase-7 expression by nuclear Wnt13 forms might be mediated by one of these transcription factors, and this hypothesis needs to be tested in further experiments.

7.3.1.2 Bim

Besides FOXOs, the transcription of Bim can be regulated by other factors. The mRNA levels of Bim was shown to be induced by cerebral ischemia in mice, while this induction was suppressed in RelA (a subunit of NF-κB) ^{CNSKO} mice; and a putative NF-κB site was then found in Bim promoter, which could be responsive to RelA; gel shift assay also confirmed the RelA binds to Bim promoter to stimulate transcription (Inta I et al. 2006). In hepatic cells, TGFβ was found to activate Bim transcription by increasing the expression of Runx1 via the mechanism of IRES, which then binds FOXO3a as a co-activator leading to the transcriptional up-regulation of Bim through FOXO binding site, revealing that both Runx1 and FOXO3a can regulate the activation of Bim transcription (Wildey GM et al. 2009). Unlike Runx1 which regulate Bim through FOXO site, Runx3

was shown to be responsible for transcriptional activation of Bim in gastric epithelial cell apoptosis induced by TGFβ by interacting with three Runx sites in human Bim promoter; one Smad site was identified from Bim promoter, which was also responsive to TGFβ-induced Bim expression (Yano T et al. 2006). Moreover, the expression of Bim was able to be induced by nerve growth factor (NGF) withdrawal in neuronal cells, because the activation of cyclin-dependent kinase (cdk) 4 de-repressed transcription factor E2 promoter binding factor (E2F), which targeted C-myb gene to induce Bim transcription via myb binding sites, so the Bim expression could be regulated during any step of cdk4-E2F-myb pathway in response to NGF withdrawal (Biswas SC et al. 2005). Besides myb, c-Jun and FOXO were found to be required for transcriptional induction of Bim in response to NGF deprivation through interacting with AP-1 site and FOXO-binding site, respectively (Biswas SC et al. 2007). The human Bim promoter was also shown to be activated by E2F factor through the interaction with a putative E2F-binding site, indicating a direct regulation of E2F in Bim transcription (Gaviraghi M et al. 2008).

Since the upreuglation of Bim expression occurred at the mRNA levels more prominently, the induction of Bim took place more possibly at transcriptional levels. Therefore, the Bim promoter is needed to define whether nuclear Wnt13 forms upregulated Bim expression through FOXO factors. If the effect of nuclear Wnt13 is in FOXO-independent manner, other factors that regulate Bim transcription, such as NF-κB, Runx family, E2F, could be considered as potential mediators for the action of nuclear Wnt13 forms.

Also, Bim and caspase-3 can be regulated post-transcriptionally. Mitogen-activated protein kinase kinase kinase kinase (MAP4K) 3 is a member of the Ste20

family of protein kinases and can be activated by UV radiation and the pro-inflammatory cytokine TNF-α, which was shown to modulate Bim expression at the posttranscriptional level dependent of its kinase activity (Lam D et al. 2009). In murine B lymphoma cell lines, anti-IgM induced prolonged activation of JNK which upregulated Bim expression posttranscriptionally, resulting in cell apoptosis (Takada E et al. 2006). Moreover, caspase-3 has been shown to undergo a unique post-transcriptionally regulation in mouse and rat during development of skeletal muscle, but not other tissues (Ruest LB et al. 2002). However, which factor is responsible for the post-transcriptional regulation of caspase-3 is still to be investigated. Thus, it is possible that the expression of caspase-3 and Bim is upregulated by Wnt13 forms at post-transcriptional levels.

7.3.2 Insights from other Wnt13 forms

Our data showed that mitochondrial Wnt13 forms had a moderate effect on increasing endothelial cell apoptosis and the expression of apoptotic factors including caspase-3, 7 and Bim, and this effect did not appear to be mediated by FOXOs, as the nuclear localization and the transcriptional activity of FOXOs in any luciferase constructs were unchanged by mitochondrial forms. One possibility is the mitochondrial forms might relay the signal from mitochondria to those nuclear transcription factors who are able to regulate caspase-3, 7 and Bim directly or indirectly. For example, under some circumstances like stress, p53 can translocate to mitochondria where it possibly interacts with mitochondrial Wnt13 forms, which might result in the activated or stabilized p53 translocating to nucleus to 1) induce caspase-7 transcription; 2) induce apoptosis through

other pro-apoptotic factors such as Noxa, Bid. Another possibility is that due to the importance of mitochondrial permeability as well as a large scale of pro-apoptotic factors located in mitochondria, the mitochondria Wnt13 forms might exert certain positive effects on 1) MOMP formation by promoting Bax translocation to mitochondria or the opening of channels like VDAC; or 2) accelerating the release of pro-apoptotic factors like cytochrome c to cytoplasm. In addition, since the amount of ROS (mainly superoxide) was increased significantly by the mitochondrial Wnt13 forms as evidenced by DHE staining, the mitochondria Wnt13 forms might affect MOMP and apoptosis through regulate ROS production. All theses proposed mechanisms need to be testified in further experiments.

However, Wnt13A showed a weak effect on EC apoptosis and a moderate increasing effect on the expression of caspase-3, 7 and Bim. Wnt13A reduced FOXO3a phosphorylation at Ser253 (an Akt/SGK site), and Wnt13A did not affect Akt phosphorylation at Ser473, suggesting that other kinases like SGK may be responsible for the decreased FOXO3a phosphorylation at Ser253. Wnt13A also increased the nuclear localization of endogenous FOXO3a; however, Wnt13A did not appear to increase FOXO activity in either FHRE-luc or SOD2 promoter –luc. Instead, it seems that the P7 fragment is more likely to be involved in the increased SOD2 transcription. The P7 fragment is featured by multiple Sp1 and AP-2 binding sites, so Wnt13A might regulate the luciferase activity of P7 through Sp1 or AP-2 factors. In addition, Wnt13A has been demonstrated to be the ligand for Fz7 receptor in BAECs (Struewing IT et al. 2007). Fz7 was also proved to interact with Wnt11 to control cell contact persistence at the plasma membrane, suggesting the cross-talk between Wnt/PCP pathway and cell-cell adhension

(Witzel S et al. 2006). Hence, it is possible that Fz7 affect JNK signaling which is a key pathway in Wnt/PCP signaling. So we propose that Wnt13A might stimulate JNK signaling through Fz7 receptor and then activate c-Jun to upregulate MnSOD expression via AP-1 site located between -1.6K and 3K of *SOD2* gene.

7.4 Clinical implications

The vascular endothelium is an active, dynamic tissue controlling many important functions, including regulation of vascular tone and maintenance of blood circulation, fluidity, coagulation, and responses to leukocytes and inflammatory factors (Gonzalez MA et al. 2003). Neovascularization and vessel regression are determined by the balance between proliferation and apoptosis of ECs, which is tightly controlled to maintain vessel homeostasis (Mallat Z et al. 2000).

During normal vessel development and remodeling, EC apoptosis have been shown to be required for vessel regression. Failure of EC apoptosis in vessel regression can cause severe pathological change. Familial exudative vitreoretinopathy (FEVR) is a hereditary ocular disorder characterized by supernumerous vascular branching due to failure of vessel regression, retina detachments and leaky vasculature (Miyakubo H et al. 1982; Masckauchán TN et al. 2006). Another human disorder, called persistent hyperplastic primary vitreous (PHPV), is caused by defective regressing of hyaloid vascular system, resulting in the persistence of structures of the primary vitreous and the development of a fibro-vascular retrolental plaque. Indeed, some components in Wnt signaling have been shown to be correlated with such diseases. For example, Fz4

mutations was found in association with familial exudative vitreoretinopathy (FEVR) (Robitaille J et al. 2002; Omoto S et al. 2004); and Fz5 retina-specific knockout mice developed a phonotype similar to PHPV, characterized by accumulation of retrolental tissue, failure of vessel regression, and abnormal retina morphogenesis (Zhang J et al. 2008). Wnt13 isoforms, especially the nuclear forms, increased the sensitivity of EC to apoptosis both at basal levels and after challenged by stimulus (LPS treatment). Moreover, the expression of Wnt13 is tightly controlled during the development of chicken eye, and Wnt13 has been shown to function in control cell fate in retina, and play critical role in the formation of laminar structure, ciliary marginal zone (CMZ) and iris/ciliary epithelium in the retina (Kubo F et al. 2003; Kubo F et al. 2009). Thus, all this information gives a hint that Wnt13 forms might play a role in vessel regression, and defects in Wnt13 forms might be involved in the pathogenesis of vascular disorders such as PHPV and FEVR.

Besides, endothelial cell apoptosis appears to be involved in other vascular disorders such as atherosclerosis and angiogenesis (Mallat Z et al. 2000; Dimmeler S et al. 2000).

Atherosclerosis is a multifactorial disorder which develops in the arterial wall in response to numerous pathological insults and results in excessive inflammatory injury and fibro-proliferative plaque (Sima AV et al.2009). An increasing size of evidence shows increased EC apoptosis in atherosclerotic plaques compared with normal tissues (Choy JC et al. 2001). In the early stage of atherosclerosis, EC apoptosis induces loss of EC number and EC integrity, leading to enhanced vascular permeability, SMC migration and increased blood coagulation (Choy JC et al. 2001). EC apoptosis may be induced by

oxLDL, CTL, cytokines, ROS or local inflammatory mediators (Sima AV et al.2009). In our research, we used pro-inflammatory LPS to challenge BAECs and EC induce apoptosis, and the nuclear Wnt13 forms further potentiate LPS-induced apoptosis by upregulating pro-apoptotic factors. Thus, two possible scenarios would be generated by Wnt13 forms: 1) more EC apoptosis in atherosclerotic lesion by Wnt13 forms might promote EC dysfunction and vascular permeability, resulting in aggravating lesion at vessel wall; 2) increased susceptibility to stimuli might help the injured cell to complete apoptosis and self-removal in a quicker pace, possibly leading to the reduction of inflammation. Therefore, whether Wnt13 is good guy or bad guy during pathogenesis of atherosclerosis needs to be confirmed in atherosclerotic models.

Angiogenesis is the process of postnatal neovasuclarization, mediated by proliferation, migration and remodeling of differentiated endothelial cells. Unlike being mainly deleterious in atherosclerosis, EC apoptosis, counteracting proliferation, has an inhibitory function in tumor angiogenesis (Dimmeler S et al. 2000), endothelial cell apoptosis is believed to be a target to interfere tumor angiogenesis. In this case, the increasing effect of Wnt13 on ECs may imply that Wnt13 forms might have inhibitory action on tumor angiogenesis. However, this hypothesis is in need of more experiments performed in endothelial cells from smaller vessels.

7.5 Future directions

Our findings reveal that the nuclear Wnt13C is regulated at translational levels during apoptosis, and the nuclear Wnt13 forms increase EC sensitivity to apoptosis by

upregulating pro-apoptotic factors such as caspase-3, 7 and Bim; also, the nuclear Wnt13 forms increase *SOD2* transcription through intron 2 element of *SOD2* gene, which is possibly mediated by FOXOs. Hence, further studies include: 1) how nuclear Wnt13C undergo regulation through uORFs; 2) whether FOXOs are responsible for the upregulation of caspase-3, 7 and Bim by the nuclear Wnt13, which can be confirmed by using promoters of caspase-3/7 and Bim; 3) whether other factors are responsible for the upregulation of caspase-3, 7 and Bim by the nuclear Wnt13 (the possible candidate regulators were described above); 4) whether the increased apoptosis by the nuclear Wnt13 is correlated in the development of diseases, which needs to be defined in disease models.

Moreover, Wnt13A and mitochondrial Wnt13 forms show less increase in EC apoptosis and the expression of apoptosis-related factors, and they do not seem to function through FOXOs. Therefore, more studies can be centered on: 1) which pathway that mitochondria forms of Wnt13 signal from mitochondria to nucleus; 2) whether Wnt13A function through Fz-7- JNK – AP-1- SOD2 pathway.

In conclusion, the findings of this research point to the interplay between Wnt13 forms and cell apoptosis. Specifically, the nuclear Wnt13C is induced at translational levels during EC apoptosis; and the nuclear Wnt13 forms increase EC susceptibility to apoptosis by upregulating the expression of pro-apoptotic factors, which suggests that the expression and functions of the nuclear Wnt13 may form a positive feedback during apoptosis, resulting in accelerating the completion of the apoptosis process (Figure 7). Therefore, the interplay between the expression and functions of Wnt13 forms, especially

nuclear forms, is important for EC homeostasis, which might also be involved in vessel development and vessel pathogenesis.

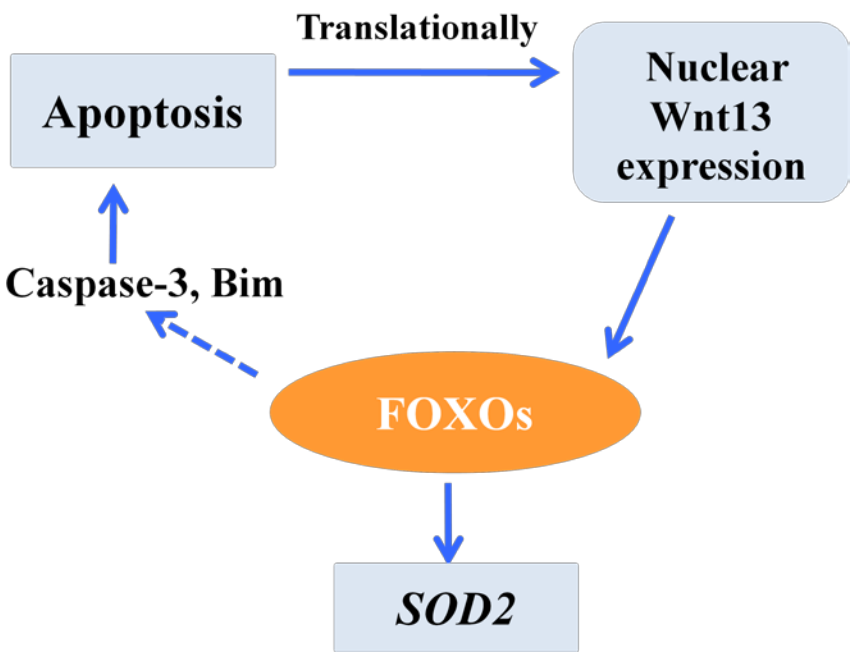


Figure 7 A possible model demonstrating the interplay between expression and functions of Wnt13 forms during cell apoptosis. Specifically, the nuclear Wnt13C is induced at translational levels during EC apoptosis. The nuclear Wnt13 forms increase FOXO activity to upregulate SOD2 expression, as well as the expression of caspase-3 and Bim leading to increased EC susceptibility to apoptosis. And this interplay between the expression and functions of the nuclear Wnt13 may form a positive feedback during apoptosis, resulting in accelerating the completion of the apoptotic program.

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PUBLICATIONS

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